HIGHLY DISABLED HERPES SIMPLEX VIRUS 1 VECTORS: APPLICATIONS IN CNS REGENERATION WITH EMPHASIS ON THE CHRONICALLY INJURED SPINAL CORD

A thesis submitted to the University of London for the degree of

Doctor of Philosophy

by

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ABSTRACT

Regeneration of injured CNS tracts poses a significant clinical challenge due to the multifactorial nature of the degenerative processes that ensues and technically complex delivery of therapeutic molecules. To circumvent these problems, highly disabled Herpes Simplex 1 (HSV1) based vectors were used to deliver NT3, BDNF and CNTF to the injured rat CNS. The ability of these vectors to promote the regeneration of corticospinal (CST), rubrospinal (RST) and optic nerve tracts was evaluated. Transgene expressing constructs were based on the backbone HSV1.pR19CMV that carries a combination of essential and non-essential gene deletions. These render it replication incompetent *in vivo* whilst allowing it to maintain transgene expression in the long term via expression cassettes that employ HSV1 latency associated transcript promoters.

Via retrograde transport, a single spinal cord inoculation results in widespread, stable transgene expression in neurons throughout the CNS, from spinal cord to hypothalamus. In spinal cord regeneration experiments, NT3-expressing vector alone failed to promote regeneration in the chronically injured CST. In combination with transplanted embryonic day-14 spinal cord segments however, it led to enhanced CST sprouting and axon elongation up to 600μm within the lesion site. In the chronically injured RST, BDNF and CNTF-expressing vectors produced no significant regeneration. In the optic nerve crush model however, both robust transduction and substantial axonal regeneration resulted from inoculation of retinal ganglion cell (RGC) targets. A combination of CNTF, BDNF, bFGF and Neurturin-expressing vectors induced regeneration of injured RGC axons in optic nerve, up to 1000 μm distal to the crush site. Further vector development led to less-disabled vectors that supported strong transgene expression and targeted spinal cord, RGCs and even CST neurons, with high efficiency. By counteracting the host's immune response, minimally disabled IL10-expressing vectors supported long-term expression of transgenes in DRG neurons following footpad inoculation. HSV1-based vectors are therefore powerful and versatile gene therapy tools for CNS regeneration.

"Ο βίος βραχύς, η δε τέχνη μακρά, ο δε καιρός οξύς,
η δε πείρα σφαλερή, η δε κρίσις χαλεπή „

(Ιπποκράτης, ¨Αφορισμοί¨)

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DECLARATION

All the work presented in this thesis is the work of Filitsa Groutsi. Contributions by other researchers are gratefully acknowledged throughout this thesis and are summarised below:

- The *in situ* hybridisation plate shown in section 3.4 (Chapter 3) was performed by Dr J. Palmer (Biovex Ltd) and is included here only as reference.

- Spinal cord surgery was carried out in collaboration with Prof P. Anderson and Dr G. Campbell (Dept. of Anatomy & Developmental Biology, UCL).

- Prof P. Anderson performed the embryonic tissue transplantation surgery shown in section 4.3.3 (Chapter 4).

- Dr M. Mason (Dept. of Anatomy & Developmental Biology, UCL) and Dr G. Campbell performed the spinal cord injection shown in Figure 4.3.5-a (Chapter 4).

- The programming for the Open-Lab software-based measurements of neuronal cell body areas described in Chapter 5, was done in collaboration with Dr G. Campbell.

- The superior colliculus and optic nerve lesions described in Chapter 6 were carried out in collaboration with Dr G. Campbell.

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GLOSSARY

AAV	Adeno-Associated Virus
ABC	Avidin Biotin peroxidase Complex
ATF3	Activating Transcription Factor 3
BDA	Biotinylated Dextran Amine
BDNF	Brain-Derived Neurotrophic Factor
BM	Basal Membrane
BSA	Bovine Serum Albumin
CAM	Cell Adhesion Molecule
CMC	Carboxy-Methyl Cellulose
CMV	Cytomegalovirus
CSF	Cerebrospinal Fluid
CSPGs	Chondroitin Sulphate Proteoglycans
CST	Corticospinal Tract
CNS	Central Nervous System
CNTF	Cilliary Neurotrophic Factor
DAB	3,3'Diaminodenzidine tetrahydrochloride
DMEM	Dulbecco's Modified Eagle's Medium
DMSO	Di-methyl Sulfoxide
DNA	Deoxyribonucleic Acid
DREZ	Dorsal Root Entry Zone
E gene	Early gene
ECM	Extracellular Matrix
EDTA	Ethylenediaminetetraacetic Acid
EF1α	Elongation factor 1α
eGFP	Enhanced Green Fluorescent Protein
ELISA	Enzyme-Linked Immunosorbent Assay
FCS	Foetal Calf Serum
FGF	Fibroblast Growth Factor
FGM	Full Growth Medium
Gal-1	Galectin –1
GAP	Growth Associated Protein
GDNF	Glial-derived Neurotrophic Factor
GFAP	Glial Fibrin-Associated Protein
HBSS	Hank's Buffered Salt Solution
HCF	Human Cellular Factor
HCl	Hydrochloric Acid
HEPES	4-(2-hydroxyethyl)-1-piperazineethanesulfonate
HMBA	Hexamethylammonium bisacetamide
HRP	Horseradish peroxidase
IL	Interleukin
IR	Immunoreactivity
IRES	Internal Ribosome Entry Site
ITR	Inverted terminal repeats
JNK	Jun N-terminal Kinase
LPR	Lipopolysaccharide
LRR	Leucine-rich repeats
LTR	Long-terminal repeats
LV	Lenti-virus
MAG	Myelin Associated Protein
MOI	Multiplicity of Infection
MOSP	Myelin Oligodendrocyte Specific Protein
mRNA	Messenger RNA
NaCl	Sodium Chloride
NaOH	Sodium Hydroxide
NCAM	Natural Cell Adhesion Molecule
NGF	Nerve Growth Factor
NgR	Nogo Receptor
NO	Nitric Oxide
nNOs	Neuronal Nitric Oxide Synthase
NT-3	Neurotrophin 3
NT-4/5	Neurotrophin 4/5
NTN	Neurturin
OC	Optic Chiasm
OD	Optical Density
OEG	Olfactory Ensheathing Glia
OMgp	Oligodendrocyte myelin glycoprotein
PBS	Phosphate Buffer Saline
PFA	Paraformaldehyde
Pfu	Plaque-forming Unit
rAAV	Recombinant AAV
rAAV2	rAAV serotype 2
rAAV2/5	rAAV pseudotyped 2/5
rh	Recombinant Human
PNS	Peripheral Nervous System
RGC	Retinal Ganglion Cells
RN	Red Nucleus
RNA	Ribonucleic Acid
RNAi	RNA interference
RST	Rubrospinal tract
SC	Schwann cell
SCG10	Superior cervical ganglion-10
SD	Standard Deviation
SEM	Standard Error of the Mean
shRNA	short hairpin RNA
siRNA	small interfering RNA
SNpc	Substantia Nigra Pars Compacta
TBS	Tween 20® Buffered Saline
TMB	Tetra-Methyl Benzidine
TNF	Tumour Necrosis Factor
Trk	Tyrosine Kinase
WHV	Woodchuck Hepatitis Virus
WPRE	Post-transcriptional regulatory element
X-GAL	β galactosidase

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CHAPTER 1.0

INTRODUCTION

1.1 Introduction

Acute injuries of the spinal cord are among the most common causes of severe disability and death following trauma. Until recently, it was thought that repair of lesioned neurons of the central nervous system (CNS) was impossible while the resulting clinical impairment was considered irreversible. The neuroscientist Ramon Y Cajal was the first to report that "*...the failure of regeneration is not an irresistible law, but a secondary outcome of a physical or chemical environment unfavourable for the growth of the sprouts*" (Ramon Y Cajal, 1928). Nearly a century later a number of studies have provided more convincing evidence that adult CNS mammalian neurons do retain their embryonic ability to mount a regenerative response but their axons are unable to cross the site of an injury, a step that is necessary in order to form synapses (Schwab and Bartholdi, 1996). Numerous studies have put forward potential hypotheses that seek to explain the mechanism behind this abortive regenerative response: firstly, the induction of axonal elongation in sprouting CNS neurons may require a significant amounts of different neurotrophic factors not readily available in the adult CNS (Berry *et al.*, 1996; Zhang *et al.*, 1998; Bradbury *et al.*, 1999; Logan and Berry, 2002); secondly, the innate ability of adult CNS axons may not be strong enough to sustain axonal elongation in the long term (Richardson and Verge, 1986; Chong *et al.*, 1996; Anderson and Lieberman, 1999); thirdly, the process of neuronal damage is in itself complex and unique to the CNS environment. Identification of key factors involved in the CNS response to injury has demonstrated that it is an inhospitable milieu for regeneration as it contains significantly potent inhibitory molecules that are either present in normal myelin (Schwab, 1996) or are upregulated following a mechanical injury (Fawcett and Asher, 1999; Pasterkamp *et al.*, 1999; GrandPre and Strittmatter, 2001; Schwab, 2002; Fournier *et al.*, 2002; Hunt *et al.*, 2002b; Sandvig *et al.*, 2004; Schwab, 2004).

In this thesis emphasis is placed on whether enabling injured CNS neurons to endogenously produce neurotrophic factors via gene delivery has a beneficial effect on axonal regeneration. A plethora of different techniques are currently under investigation for their ability to deliver therapeutic genes to the lesioned

CNS and tracts of the spinal cord in particular. One gene delivery technique, under investigation in this study, was based on the use of disabled Herpes Simplex Virus 1 (HSV1) vectors. The construction of neurotrophic expressing HSV1 based vectors and their potential as gene therapy tools for amelioration of the neurodegenerative effects of injury in the corticospinal, rubrospinal tracts in the spinal cord as well as the optic nerve, was investigated.

1.2 Spinal cord injury

Spinal cord injury can be defined as "*... a lesion to the spinal cord that results in partial or complete paralysis*" (Johnson and Griffin, 1997). It can be the result of acute trauma, caused by a direct insult to the spine or disease such as vertebral tumours, polio and motor-neuron disease. This study focused on the factors involved in traumatic spinal cord injury and will attempt to examine the manner in which these contribute to the disabling neurological impairment that follows. Overall, the type and severity of the neurological deficit depends on the way that spinal elements respond to the force of the injury (Hardy, 1975).

1.2.1 Incidence and prevalence of spinal cord injury

Spinal cord injury is one of the leading causes of mortality following acute trauma but there are significant variations in incidence and prevalence between western and developing countries (Ackery *et al.*, 2004). There is a lack of countrywide statistics taking into account all the different spinal cord injury rehabilitation centres in the UK. The last peer-reviewed statistical report specific to the UK was published in 2005 (Amin *et al.*, 2005). The authors report a four-year retrospective review between March 1988 and March 2003 on all new spinal cord injury admissions to the Royal National Orthopaedic Hospital in Stanmore. According to this survey, spinal cord injury primarily affects adults with an average age of 38.6 years for men and 41.8 for women. Out of the total number of patients affected, 82% were male and 18% were female while the leading causes of traumatic spinal cord injury were predominantly: motor vehicle accidents (35.9%), falls (58.6%), sports injuries and gunshot wounds (5.5%).

The most common cause of death amongst spinal cord injury patients is respiratory failure while advances in acute trauma management have enhanced prevention of renal failure (Burns, 2007). An increasing number of spinal cord injury patients are dying due to other causes such as cancer or cardiovascular disease, similar to that of the general population. Overall, 85% of patients who survive the first 24 hours after trauma are still alive 10 years later. However, mortality rates are significantly higher during the first year after injury than in subsequent years. In 2004 the National Spinal Cord Injury Statistical Centre (NSCISC, University of Alabama, http://www.spinalcord.uab.edu, PMID: 16396388) reported an annual incidence of 40 cases per million population in the USA, with a prevalence of 250,000 cases. In this study the most common causes of spinal cord injury were industrial injuries (34.5%) and road traffic accidents (33.1%) (Jackson *et al.*, 2004).

1.2.2 Summary of Clinical Manifestation of Spinal Cord Injury

To accurately evaluate the extent of neurological impairment as a result of spinal cord injury, assessment of lesions in motor and sensory pathways is required. Loss of function in myotomes and/or dermatomes can give an indication of the spinal nerves involved and an anatomical reference to the level of the spinal cord injury. Due to the complexity in the distribution of the sensory and motor pathways along the spinal cord, more than one tract can be involved in a single injury. Hence, consideration of reflex actions, functions controlled by spinal nerves in the viscera and the distribution of the neurological impairment should always be considered in conjunction. A summary of the clinical manifestation of spinal cord injury with relation to the segment of the spinal cord affected is given in Table 1.2.2-a. However, certain features of neurological function after an acute injury provide significant assistance in pin-pointing the tracts affected (Hardy, 1975; Guthkelch, 1996; Rolak, 2004; Snell, 2004).

Spinal Cord	Sensation Affected	Motor Function Affected	Summary of Clinical Features
C1-C3	Face, Scalp Neck	Head, Neck	**Changes in Voluntary Power** Muscle control is impaired. Clinical examination can give an indication of the cervical spinal cord segment affected. However, muscle innervation is derived from at least two or three segments. In addition, the majority of spinal cord lesions usually involve more than one segment. Hence, the upper level of motor paralysis is difficult to define.
C4	Shoulder cape	Diaphragmatic control	**Decrease in Sensibility (sensory neurons)** Cutaneous areas supplied by adjacent spinal nerves overlap to a certain degree. Thus, there is little sensory loss following the disruption of a single spinal nerve root. However, spinal cord injury rarely involves damage of only a single spinal nerve.
C5-C6	Thumb, index and middle finger	Deltoid Biceps	**Changes in Reflex activity (Spinal Shock)** Reflex activity is altered above and below the injury site. In cases of complete loss of reflex activity (Spinal shock), all somatic & visceral activity is lost.
C7-C8	Middle & Small Finger	Triceps, Wrist Flexors, Finger Extensors Finger Flexors	**Difficulty in Respiration** Lesions of the third and fourth cervical segments affect control of respiration. A complete lesion is not compatible with unsupported life.
T1-T10	Trunk, progressively down to the navel	Intercostals Spine Extensors	**Dominant or transient Pain** Pain is associated primarily with fractures and its intensity varies considerably. Absence of pain has been observed in cases of traumatic paraplegia.
T11-T12	Inguinal Ligament	Rectus Abdominis	**Impaired Respiration** Difficulties in respiration are primarily caused by paralysis of the intercostal muscles although associated soft tissue injuries, such as rib fractures, can result in hemothorax and lung function impairment. **Impairment of the Abdominal Viscera** Loss of rectal sensation and inability to pass urine.
L1-L2	Anterior Thigh Medial Thigh	Iliopsoas Sartorius & Adductors	**Dominant or transient pain** **Changes in Reflex activity** Tendon reflexes can be lost but on the sensory side sensation in the perianal are may be retained.
L3-L5	Medial calf Dorsum of Foot	Quadriceps	
S1-S4	Lateral aspects of Foot Posterior Thigh Perianal region	Toe flexors Extensors Bowel & Bladder Sphincters	**Paralysis of the buttock & foot muscles** **Paralysis of urethral & anal sphincters** **Changes in sensation of the genitalia** Patients with sacral fractures suffer impairment or loss of sexual ability.

Table 1.2.2-a: Clinical Manifestation of spinal cord injury.

(Adapted from Rolak 2004).

1.3 Neuronal responses to axotomy

Mechanical trauma, compression or ischemic conditions can result in axotomy, which involves a cascade of morphological and cellular changes. The extent of axotomy is a combination of the immediate disruption caused by the insult itself (primary axotomy) as well as the impact of the delayed cellular reactions that take place around the lesion site (secondary axotomy) (Blesch and Tuszynski, 2002). This does not lead just to the breakdown of a communicating link between the immediately affected neurons. The degenerative reaction initiated results in retrograde changes that can lead to variable degrees of deficit, ranging from transient alterations in axonal physiology to permanent atrophy and cell death (Ramon Y Cajal, 1928; Lieberman, 1971; Brodal, 1981; Kreutzberg *et al.*, 1989; Berry *et al.*, 1999). Generally, groups of neurons that are similar in function and physiology tend to respond to axotomy in a similar manner. This feature is thought to be a reflection of the intrinsic properties of the same group of neurons as well as similarities in the extracellular matrix (ECM) characteristics that these neurons have to adapt to (Sofroniew, 1999). Unlike the Peripheral Nervous system (PNS), the CNS does not exhibit the same ability to regenerate after axotomy. The molecular mechanisms behind this difference are slowly becoming clear but the prevailing theory suggests that the CNS regenerative failure is thought to be due to both intrinsic and extrinsic factors (Grill and Tuszynski, 1999). Intrinsic, referring to the innate ability of CNS neurons to mount a regenerative response and reinitiate axonal growth (Bregman and Kunkel-Bagden, 1988; Anderson and Lieberman, 1999; Horner and Gage, 2000; Hunt *et al.*, 2002a) and extrinsic referring to features of the extracellular environment that render any such attempt abortive (Logan *et al.*, 1994; Fawcett and Asher, 1999; Logan and Berry, 2002; Sandvig *et al.*, 2004; Raisman, 2004). In the sections that follow, differences between PNS and CNS neurons and the intrinsic and extrinsic factors that govern their responses will be examined[1]. Emphasis is, placed on studies that focus on the lack of regeneration following a spinal cord axotomising lesion.

[1] The exact responses of the corticospinal, rubrospinal and optic nerve tracts are examined in great detail in Chapters 4, 5 and 6 of this thesis respectively.

1.3.1 PNS neurons – cellular responses to axotomy

PNS neurons of adult mammals are capable of a higher degree of regeneration than their CNS counterparts. Despite the fact that these regenerative responses do eventually lead to some recovery of function, this is achieved via a slow and error-prone process that can lead to aberrant synapse formation clinically manifested as non-specific neuropathic pain (for a review: Grill and Tuszynski 1999).

Following axotomy of adult DRGs and motor neurons, a degenerative process termed Wallerian degeneration occurs (Waller, 1850). This is an apoptosis-like process intrinsic to the axon itself (Glass *et al.*, 1993; Stoll *et al.*, 2002) that describes the degeneration of the distal axonal stump. This degenerative process is a rapid, wave-like process (Lubinska, 1982) that occurs at different intervals for the axons comprising a mixed peripheral nerve (Beirowski *et al.*, 2005). Within hours following transection, the myelin sheath begins to swell and degenerate with myelin breaking down into fatty material which by the end of the first 10 days following axotomy becomes oily droplets surrounding the axons (Lassmann *et al.*, 1978). This debris is removed by phagocytosing Schwann cells and macrophages, which surround the disintegrating axons (Stoll *et al.*, 1989; Liu *et al.*, 1995; Fernandez-Valle *et al.*, 1995). Macrophages and other leukocytes start to invade the distal axon between 3 and 5 days after the lesion, reaching a peak between two and three weeks later (Perry and Brown, 1992; Liu *et al.*, 1995; Avellino *et al.*, 1995). The axon becomes granular and the cytoskeleton starts to disintegrate as the microtubule and neurofilament proteins disengage. It is thought that it is this disruption in the microtubule system and the severe compromise it causes in the anterograde axonal transport mechanism of the neuron that exacerbates axonal degeneration (Bignami *et al.*, 1981). This is based on findings that Wallerian degeneration can be initiated with the use of microtubule disrupting agents such as colchicine (Singer and Steinberg, 1972). Within 24 hours following injury, axons of the proximal stump become swollen due to the arrival of newly synthesised cytoskeletal components and organelles that are transported down to the site of growth cone formation (Meller, 1987; Fournier and McKerracher, 1995).

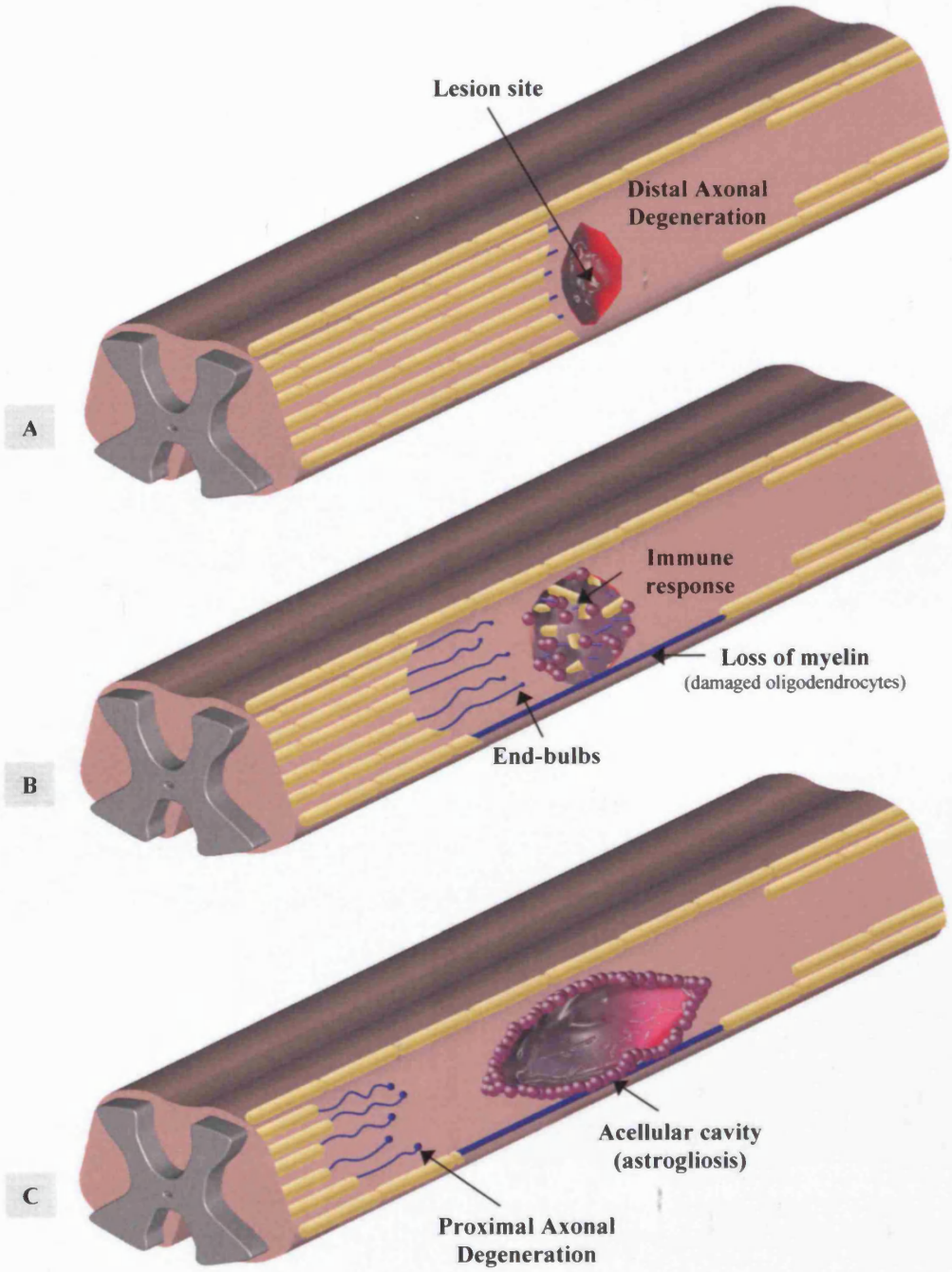
Soon after the lesion Schwann cells undergo hypertrophy and start to invade the vacant areas previously occupied by the now disintegrating myelin and axons (Liu *et al.*, 1995). In the distal axon segment, Schwann cells divide and align themselves along pre-existing basal lamina forming columns termed Bands of Büngner (Ide, 1996) which act as growth pathways for the growth cones arising from regenerating axons in the proximal stump (Grill and Tuszynski, 1999). Schwann cells comprising the Bands of Büngner produce a number of factors that aid the process of axonal elongation. Firstly they produce adhesion molecules such as L1 and neural cell adhesion molecule (NCAM) (Nieke and Schachner, 1985; Tacke and Martini, 1990) to which growth cones can attach. Secondly, they synthesise ECM protein components such as laminin, fibronectin (Salonen *et al.*, 1987; Wang *et al.*, 1992a; Wang *et al.*, 1992b; Fawcett and Asher, 1999) and tenascin-C (Martini *et al.*, 1990) which stimulate axonal attachment and elongation. Thirdly, in response to cytokines such as interleukin (IL) 1 (Lindholm *et al.*, 1987; Heumann *et al.*, 1987), they secrete trophic factors such as nerve growth factor (NGF), brain-derived neurotrophic factor (BDNF), basic fibroblast growth factor (bFGF) and glial cell line-derived neurotrophic factor (GDNF) (Tacke and Martini, 1990; Acheson *et al.*, 1991; Meyer *et al.*, 1992; Funakoshi *et al.*, 1993; Liu *et al.*, 1995; Hammarberg *et al.*, 1996).

1.3.2 CNS neurons - cellular responses to injury

The environment of CNS axons is structurally different from that of PNS neurons. A schematic representation of the organisation of neuronal and non-neuronal cells of the CNS is shown in Figure 1.3.2-b (page 31). Firstly, CNS axons are not physically separated like peripheral nerves but are instead organised into anatomically distinct tracts of similar function e.g.: dorsal columns. Secondly, oligodendrocytes (the CNS myelinating cells) differ from Schwann cells in that a single cell can myelinate a number of axons at the same time (Grill and Tuszynski, 1999). A mechanical injury alters the electrophysiological properties of the neuronal cell membrane. Such changes, result in a sudden influx of Ca^{2+} and Na^{+} ions that can reach the cell body (Emery *et al.*, 1991; George *et al.*, 1995; Ziv and Spira, 1997). This increase in

intracellular calcium levels activates intracellular proteases such as calpain, which by digesting cytoskeletal components causes the degradation of axons at the lesion site (Newcomb *et al.*, 1997; Posmantur *et al.*, 1997; Kampfl *et al.*, 1997). The initial sudden influx of Ca^{2+} ions following the mechanical damage has also been shown to induce the dedifferentiation of axonal segments into growth cones (Ziv and Spira, 1998). As is the case in the PNS, damage to CNS intrinsic neurons causes the distal axon to undergo Wallerian degeneration. Even though the proximal stump and associated cell body usually survives, left unaided fails to initiate a regenerative response. The cellular events initiated after a traumatic spinal cord (focal) lesion, result in the progressive degeneration of neuronal cells and can be divided into an immediate, an intermediate and a late phase (Summarised in Figure 1.3.2-a).

There is a substantial difference in the cellular responses that occur in the PNS and CNS following axotomy (reviewed in Grill and Tuszynski, 1999). Unlike the case in the PNS, there are no structures with a function equivalent to the Bands of Büngner that could support the elongation of growth cones. Responses in the CNS are slow and not as efficient as those seen in the case of a PNS injury. Following an acute injury in the PNS, the majority of the myelin and axonal debris is removed by 30 days after the lesion, in the CNS the debris is still present in large amounts by the end of 90 days after spinal cord injury (George and Griffin, 1994) and at least 42 days after an optic nerve lesion (Ludwin and Bisby, 1992). This delay could be a reflection of the properties of glial cells, that support neuronal function. Unlike Schwann cells, oligodendrocytes do not have the ability to phagocytose fragmented myelin debris (Bignami and Ralston, 1969). Oligodendrocytes do not align themselves into linear structures such as the Bands of Büngner as Schwann cells do following a PNS lesion. In addition, due to the presence of the blood-brain barrier, peripheral macrophages are not so easily recruited in the CNS. Therefore, the main phagocytic activity is carried out by the activation of CNS-resident microglia (Figure 1.3.2-c, page 32) (Stence *et al.*, 2001). Activated microglia have number of significant functions including antigen presentation via their MHC class II and complement-mediated phagocytosis (George and Griffin, 1994; Aloisi, 2001).


Lesion site
Distal Axonal Degeneration
A
Immune response
Loss of myelin
(damaged oligodendrocytes)
End-bulbs
B
Acellular cavity
(astrogliosis)
Proximal Axonal Degeneration
C

	Within the Lesion Site	Outside the lesion site
Immediately A	• Axons are transected • Astrocytes & oligodendrocytes destroyed • Local blood supply interrupted • Inward leakage of serum components	Outward leakage of serum components from the lesioned area into surrounding tissues
Hours / Days B	• Degeneration of cut axons • Accumulation of myelin debris • Astrocytes produce laminin and proteoglycans • Astrocytes produce truncated neurotrophin receptors • Infiltrating macrophages & microglia • Production of inflammatory cytokines (IL-1, IL-6) • Leakage due to compromised blood-brain barrier	Tissue damage area expands Death of neurons Astrocytes begin to wall-off the injury site Inflammatory response spreads to surrounding tissue
Weeks / Months C	• Formation of necrotic tissue • Formation of acellular cavity • Cavity extends outwards from the site of lesion • Inflammatory cells persist in low numbers • Failure of axon regeneration – acellular cavity • Dystrophic present in fluid cavity	Astrocytic response scar surrounding the lesion Failure of axon regeneration at the glial scar Cavity is walled off from surrounding viable tissue

Figure 1.3.2-a: Summary of cellular changes following spinal cord injury.

Yellow segments and blue lines represent myelin and axons respectively. Adapted from Fitch and Silver (1999).

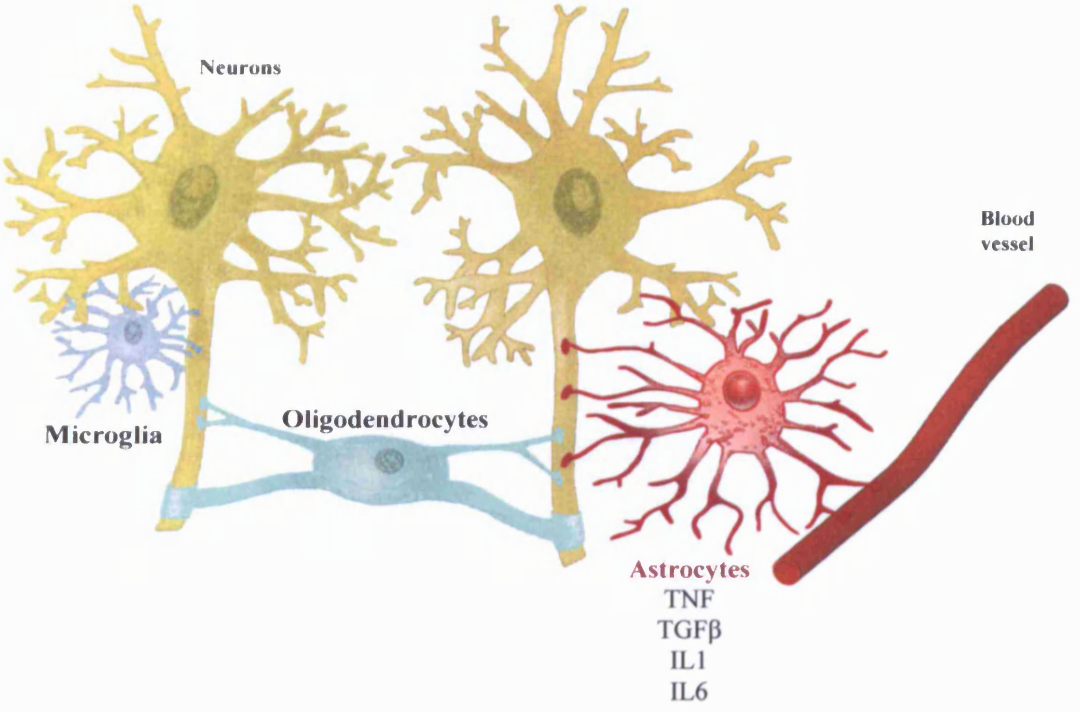
In addition they are capable of expressing neurotrophins at least *in vitro* (Nakajima *et al.*, 2001) and they reduce glutamate toxicity by scavenging glutamate (Nakajima and Kohsaka, 2001). Microglial activation is prominent by three days following an axotomising lesion after which it declines. Three weeks later, macrophage activity reaches its peak (George and Griffin, 1994). This two week delay in the onset of any significant phagocytic activity further prolongs the accumulation of debris (George and Griffin, 1994). Some reports though point out that this delay in activation varies between different rodent strains (Shaked *et al.*, 2004). Astrocytes are also instrumental in maintaining the correct environment around normal CNS neurons. These cells reside throughout the CNS and are responsible for maintaining an ionic balance in the neural microenvironment by removal of excess potassium ions (Risau and Wolburg, 1990; Wolburg *et al.*, 1994; Grange-Messent *et al.*, 1996). They also prevent excitotoxicity by metabolising extracellular glutamate, high levels of which can lead to neural death, and by releasing glutathione which may have a function in protecting neurons against free radical damage (Lee *et al.*, 1992; Schousboe *et al.*, 1993; Sonnewald *et al.*, 1997). In addition, they mediate an inflammatory/immune response by release and concomitant response to immune mediators such as IL1, major histocompatibility complex (MHC) class II or transforming growth factor (TGF) β (Frank *et al.*, 1986; Stoll *et al.*, 1989; Morris and Esiri, 1991; Aranguez *et al.*, 1995; Farina *et al.*, 2007). Cytokines around the lesion site can contribute to the overall inhospitable nature of the injured CNS. They have been shown to modulate the ability of cultured astrocytes to provide a supportive environment to regenerating CNS axons. For example, *in vitro* the combination of IL1 and bFGF promotes the migration and differentiation of oligodendrocyte progenitors and therefore promotes CNS re-myelination while interferon gamma and TGFβ have the opposite effect (Fok-Seang *et al.*, 1998). It is therefore not surprising that these cells are thought to play a significant role in the overall response to injury and it was initially thought that they are the main contributors to the inhospitable for axonal regeneration nature of the CNS environment by creating a physical barrier to any regenerating neurons in the form of a glial scar (Reier and Houle, 1988). A large number of studies have attempted to establish the mechanism behind their involvement in the failure of

CNS neurons to regenerate. One of the experimental models that have been used for this type of study is based on the use of dorsal root injury (rhizotomy). This model focuses on the events that occur at the dorsal root entry zone (DREZ), the interface between PNS and CNS. Injured DRG neurons are not capable of crossing the DREZ in order to re-establish connections with their target CNS neurons (Liuzzi and Lasek, 1987; Chong *et al.*, 1999) even when their regenerative potential is increased by a preconditioning lesion (Chong *et al.*, 1996). Upon contact with the CNS astrocytic processes, regenerating DRG axons either stop regenerating (Liuzzi and Lasek, 1987) or are induced to turn back on themselves and redirect their growth towards the cell body (Zhang *et al.*, 2001). This could be due to either the astrocytes and the potentially inhibitory molecules they secrete in the DREZ (Pindzola *et al.*, 1993; Zhang *et al.*, 2001; Beggah *et al.*, 2005; Zhang *et al.*, 2006) or due to the lack of trophic signals present on these cells (Zhang *et al.*, 2000).

Very quickly following an axotomising injury, astrocytes hypertrophy and increase in volume via a process termed cytotoxic oedema. The astrocytic hypertrophy is associated with an increased production of intermediate filaments, which manifests as an increase in glial fibrillary acidic protein (GFAP) immunoreactivity (Barrett *et al.*, 1981; Eng, 1985; Berry *et al.*, 1996; Zhang *et al.*, 1997). The cause of this is not fully known but astrocyte swelling is attributed to elevated levels of potassium, glutamate, fatty acids and free radicals at the site of injury (Eng *et al.*, 2000). There is a notable increase in their proliferating state termed reactive gliosis in an attempt to "wall off" the lesion site (Figure 1.3.2-a, page 27) (Silver and Miller, 2004). Unlike in a peripheral nerve injury where Schwann cells can be found within the lesion site, GFAP immunoreactivity patterns indicate that astrocytes surround the borders of the lesion but do not enter the core of the lesion site (Farooque *et al.*, 1995). In addition to reactive gliosis and cytotoxic oedema, astrocytes also participate in the initiation of a local inflammatory response. This involves the production of inflammatory cytokines (Figure 1.3.2-b, page 31), which in turn leads to the activation of resting microglia. In the case of lesions that leave the dura intact, the glial scar formed is primarily composed of astrocytes. In contrast, when the

lesion is severe enough to compromise the blood-brain barrier the glial scar is formed by astrocytes as well as a significant number of connective tissue elements such as fibroblasts (Silver and Miller, 2004). This creates fluid-filled cysts that quickly become acellular by an unclear process involving both apoptosis and necrosis (Beattie *et al.*, 2000). There is evidence to suggest that there are some differences between different species with regards to the cellular consistency of the cysts that follow an injury in the spinal cord. For example, the cysts formed in the rat contusion injury model are largely acellular compared to the cysts forming in a similar injury model in the mouse which are filled primarily with connective tissue (Inman and Steward, 2003). Cysts are formed at the site of lesion, by infiltrating peripheral macrophages, activated microglia and serum components leaking into the primary lesion area from the compromised blood-brain barrier. It has been demonstrated that the blood-brain barrier remains "leaky" to blood and other serum components for up to two weeks following a CNS lesion (Preston *et al.*, 2001).

As time progresses, necrosis occurs in the fluid filled cavity and degeneration extends both in a lateral and rostro-caudal manner. Such cavitations make the regenerative process completely impossible, as axons are unable to grow in an acellular environment (Fitch *et al.*, 1999; Silver and Miller, 2004). It has been suggested that the purpose of the astrogliosis is actually protective for the animal as a whole. It is thought that the accumulating astrocytes can repair the blood-brain barrier and limit the infiltration of peripheral macrophages in this highly sensitive immune privileged site. This would limit further degeneration by preventing the development of an overwhelming inflammatory response (Bush *et al.*, 1999; Silver and Miller, 2004; Faulkner *et al.*, 2004). It has been proposed that reactivating astrocytes, found distally to the lesion site, may support regenerating axons prior to the development of the physically impeding scar (Tom *et al.*, 2004; Okada *et al.*, 2006). The formation of the scar may be important to the survival of the animal but comes at the expense of long-distance regenerative capacity (Silver and Miller, 2004). When CNS axons encounter the glial scar their morphology changes (Ramon Y Cajal, 1928), axonal growth cones collapse and develop into dystrophic end-bulbs.



Cytokine	Effect
CSF	• GM-CSF, IL-3, CSF-1, trigger the activation and proliferation of microglia. • IL-3 expression by astrocytes acts as a chemotactic agent for microglia. • GM-CSF & CSF-1 cause the inhibition of class II MHC expression from microglia.
TNF-α	• Enhances the permeability of endothelial cells and facilitates migration of neutrophils, monocytes and lymphocytes at the site of injury. • Increases the production of class I and class II MHC molecules. • Stimulates microglia to produce: IL-1, IL-6, CSF & TNF-α
IFN-γ	• Enhances the expression of class I and class II MHC. • Enhances the activation of microglia to a phagocytic state.
IL-1	• Induces the production of secondary inflammatory metabolites • Induces the production of other cytokines: TNF-α, IL-6, TGF-β, CSF, IL-1 itself.
IL-6	• IL-6 produced by astrocytes induces their proliferation.
TGF-β	• TGF-β is a chemotactic agent for astrocytes and microglia.

Figure 1.3.2-b: Interaction between CNS neurons & glia in the injured spinal cord.

Interaction of spinal cord neurons, astrocytes, oligodendrocytes and CNS microglia. The table above summarises the cytokines that are produced by CNS astrocytes in response to an axotomising lesion.

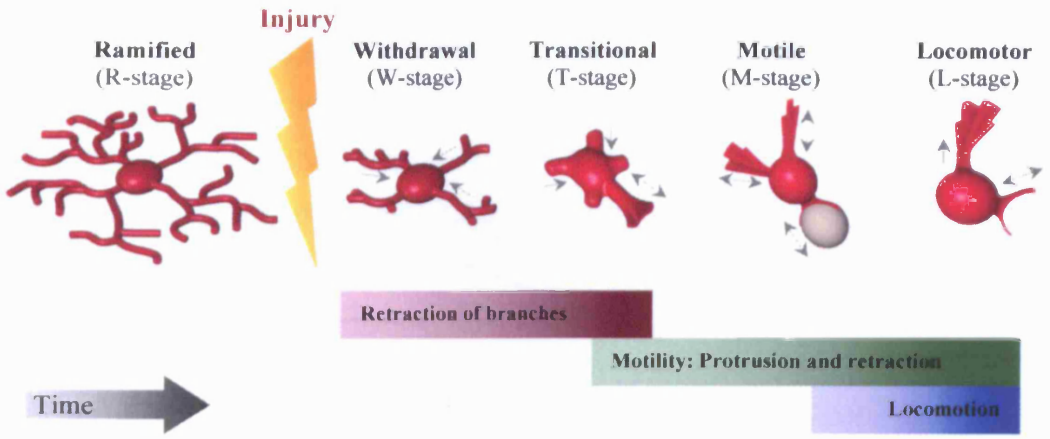


Figure 1.3.2-c: Microglial cell activation.

Resting microglia are highly ramified cells with ≈50 μm long branches (R-stage). Following the arrival of an activating signal, the ramified branches begin to withdraw at a rate of 0.5-1.5 μm/min (W-stage). At the transition stage (T-stage) the old processes are still being withdrawn while new protrusions begin to emerge. In the motility stage (M-stage) the newly formed protrusions repeatedly protrude and retract at a rate of 4 μm/min. It is at this stage that microglia begin to contact adjacent cells. During the locomotor stage, they continue to come into contact with neighbouring cells as they translocate through the tissue at a rate of 118 μm/min. (Adapted from Stence, 2001).

Growth cone collapse takes place when regenerating CNS axons come into contact with myelin components and oligodendrocytes present in the glial scar (Bandtlow *et al.*, 1990; Burden-Gulley *et al.*, 1995). For many years it was thought that once axons develop end-bulbs, it is impossible to reinitiate any regeneration. However, this was later disproved when it was shown that chronically injured rubrospinal axons which had developed dystrophic end-bulbs retain their ability to regenerate into a peripheral nerve graft placed within the lesion site (Houle, 1991). Further research into the structure of neuronal end-bulbs has confirmed at the molecular level that despite the fact they are termed dystrophic and appear incapable of any motility, these structures remain highly active, constantly altering their cytoskeleton (Silver and Miller, 2004).

1.4 Characteristics of the non-regenerative nature of CNS neurons

Unlike the vigorous response mounted from injured PNS neurons, the response seen by intrinsic CNS neurons following a lesion of similar severity and nature is characteristically abortive. It has been suggested that this feature may be a reflection of the importance of maintaining the integrity of the highly specialised neuronal circuits of the CNS (Larner, 1995). Specifically for spinal cord intrinsic neurons, axons are capable of collateral sprouting proximal to the lesion. However, left unaided, these growth cones quickly become atrophic, sprouting ceases and elongation arrests prematurely. From early on, researchers attempted to shed some light on whether this was due to the intrinsic inability of CNS neurons to mount a regenerative response or whether it was down to the properties of the CNS milieu that did not permit for such a response to be sustained.

A series of experiments hinted to the fact that the main limitation to successful CNS regeneration was the presence of inhibitory factors in the extracellular environment. Firstly, Ramon Y Cajal and his student Tello (Ramon Y Cajal, 1928) demonstrated nearly 100 years ago that it is possible for lesioned CNS neurons to regenerate when placed in an environment that favours regeneration like that of the PNS. In their experiment they placed a segment of a peripheral

nerve into the cortex of adult rabbits. Four weeks later they noted that bundles of injured putative cerebral axons had extended into the transplant. Due to limitations of the technique at the time (silver staining) it was not possible to prove beyond doubt that the regenerating axons seen entering the graft were those of regenerating CNS fibres and this experiment was treated with scepticism until the early eighties. Then, the experiment was repeated by others but this time the graft was placed either in the brain (Benfey and Aguayo, 1982; Benfey *et al.*, 1985) or in the thoracic spinal cord (David and Aguayo, 1981; Richardson *et al.*, 1982) and more accurate retrograde labelling techniques were used to identify regenerating axons as those of CNS neurons. These experiments supported the earlier claims made by Ramon Y Cajal and demonstrated that regenerating CNS axons could extend for several mm into the autologous graft. Despite the obvious regeneration, only very few regenerating fibres grew through the lesion site and into the distal spinal cord where they quickly arrested (David and Aguayo, 1981).

In a later set of experiments Berry and colleagues tested the ability of a similar peripheral nerve to stimulate the regeneration of axotomised adult retinal ganglion cells (RGCs) of the rat optic nerve (Berry *et al.*, 1986). They showed that in the optic nerve paradigm the key factor that led to RGC axonal regeneration was the Schwann cells which through the Bands of Büngner were providing the same support to CNS growth cones as they did for the axotomised peripheral axons (Berry *et al.*, 1988; Hall and Berry, 1989). However, having a Schwann cell enriched microenvironment does not promote the same regenerative outcome in all subgroups of CNS neurons. For example, a peripheral nerve graft fails to promote the regeneration of either corticospinal or Purkinje axons, indicating that the presence of Schwann cells is not always sufficient to enable axonal regeneration (Richardson *et al.*, 1982; Anderson *et al.*, 1998; Chaisuksunt *et al.*, 2000; Chaisuksunt *et al.*, 2003). This further suggests that there are inherent differences between different classes of CNS neurons that affect their ability to respond to axotomy.

1.4.1 Cell body responses of axotomised CNS neurons

The response to axotomy is not universal to all CNS neurons but depends on the group of neurons affected. However, the manner in which a single group of neurons respond reflects their inherent regenerative potential. One such factor is the age of the injured animal. For example, corticospinal tract neurons, perish when axotomised in the early postnatal stage but survive when axotomy is carried out in two-week old animals hinting that these neurons die if axotomy occurs prior to them innervating their targets (Lieberman, 1971; Merline and Kalil, 1990; Rossiter *et al.*, 1996). Age related responses to axotomy could reflect changes in either the availability or the requirements for growth factors that change as CNS neurons mature.

Another factor that has been proposed to play a part in the ability of CNS neurons to regenerate is the distance of the lesion site from the neuronal cell body. When an axotomising lesion in the spinal cord leads to the degeneration of the damaged axon back to a point of significant collateral branching, then the neuron is able to survive this injury (Ramon Y Cajal, 1928; Lieberman, 1971). In addition, the CNS neurons that were found to be regenerating into a transplanted peripheral nerve graft were found to have their somata very close to the lesion site (Richardson *et al.*, 1982). However, studies into the regenerative ability of axotomised RGC and Purkinje neurons revealed that they still survived axotomy even though the distance of the site of injury from the cell body was not interrupted with any collateral branching, thus suggesting that the presence of collaterals was not a determining factor in CNS neuronal survival and subsequent axonal regeneration (Sofroniew, 1999). It has also been suggested that the longer the distance of the site of injury from the cell body, the more difficult it is to retrogradely transmit injury-related signals such as cytokines and growth factors produced in the vicinity of the lesion. This hypothesis was postulated by a number of studies examining the effects of axonal injury on the dynamics of neuronal transport. It was noted that rubrospinal neurons axotomised at the C3 level retrogradely transported tracer dyes faster than when the axotomy was carried out at the T10 level (Tseng *et al.*, 1995). This pattern appears to correlate with the expression of growth associated genes in this tract.

Specifically, it was found that the expression of growth associated protein 43 (GAP43), laminin, tubulin and neurofilament-M is increased when axotomy occurs at the cervical but not thoracic level (Fernandes *et al.*, 1999). Similarly, corticospinal neurons are able to upregulate the expression of GAP43 when axotomy is performed in the sub-cortical white matter but this does not occur when the lesion is inflicted at the more distant level of the pyramidal decussation (Kalil and Skene, 1986; Bisby and Tetzlaff, 1992). Finally, in the case of the optic nerve, GAP43 was only found to be upregulated when RGCs were axotomised at just 3 mm from the retinal layer (Doster *et al.*, 1991).

In addition to the importance of the retrograde axonal transport, the rate of anterograde transport of cytoskeletal components is of equal significance for the repair of the severed axon. Transport of cytoskeletal components such as actin, tubulin and neurofilament, occurs via the slow component b (Scb) system in the CNS and via the slow component a (Sca) system in the PNS (Fournier and McKerracher, 1995). Following axotomy in the PNS, the rate of Sca transport increases to match the rate of axonal elongation (McKerracher and Hirscheimer, 1992). In contrast, in axotomised RGCs, the rate of Scb transport decreases from 0.5 mm/day to 0.06 mm/day (McKerracher *et al.*, 1990b). However, when RGCs are placed next to a peripheral nerve graft, they extend new processes into the graft at a rate of 1 mm/day following injury suggesting that when aided, regenerating CNS neurons can also increase the activity of Scb system in order to support the demands of axonal elongation (McKerracher *et al.*, 1990a).

1.4.2 Gene expression in axotomised CNS neurons

For CNS axonal regeneration to translate into functional restoration, neurons must be able to firstly survive the initial mechanical disruption, secondly sprout and thirdly elongate their axons in order to reach their targets. Several molecules have been implicated in stimulating axonal growth pathways, including GAP43, c-Jun and activating transcription factor 3 (ATF3), which will be briefly discussed below. Note that the patterns of expression of these molecules with regards to the corticospinal, rubrospinal and optic nerve tracts are discussed in more detail in the relevant chapters of this thesis.

GAP43 expression

GAP43 is a 43 kDa, phosphorylated protein that is highly upregulated during development (Strittmatter *et al.*, 1995; Benowitz and Routtenberg, 1997; Zhu and Julien, 1999). Its expression is re-initiated in regenerating PNS neurons and sprouting CNS neurons (Oestreicher *et al.*, 1994; Benowitz and Routtenberg, 1997; Grill and Tuszynski, 1999). GAP43 is a substrate for Protein Kinase C (PKC) and it localises to axonal growth cones where it is involved in the reorganisation of the neuronal cytoskeleton (Akers and Routtenberg, 1985; Benowitz and Routtenberg, 1997). The importance of GAP43 in axonal growth has been examined in a variety of *in vitro* and *in vivo* experiments. For example, the addition of NGF to phaeochromocytoma (PC12) cells, results in the upregulation of GAP43 expression and neurite extension (Federoff *et al.*, 1988). Overexpression of GAP43 in PC12 cells increases the number of neurites produced (Yankner *et al.*, 1990) whilst the use of antisense oligonucleotides to GAP43 mRNA reduces the number of neurites produced (Jap Tjoen *et al.*, 1992). When GAP43 antisense oligonucleotides were used on primary cultures of chick DRGs, there was a reduced number of neurites produced. The neurites produced had smaller and more unstable growth cones present and they were far more susceptible to inhibition when exposed to the inhibitory effects of CNS myelin-derived liposomes compared to neurons that expressed GAP43 as normal (Aigner and Caroni, 1993; Aigner and Caroni, 1995). However, there are some reports that tend to doubt the direct association of GAP43 expression with axonal elongation post injury. For example it has been shown that GAP43 deficient PC12 cells were capable of differentiation and axonal extension in response to NGF (Baetge and Hammang, 1991). The difference between these *in vitro* experiments could be due to the fact that GAP43 may not be an essential prerequisite for the initiation of neurite outgrowth but its presence is required in order for this response to be sustained (Baetge and Hammang, 1991; Aigner and Caroni, 1995). Further evidence that support this notion comes from a series of experiments on two different strains of transgenic mice that lack the ability to produce GAP43 (Strittmatter *et al.*, 1995; Zhu and Julien, 1999). Both strains appear to have significant developmental defects, especially in the synaptic architecture of the visual system. These experiments reinforce the fact that even

in the total absence of GAP43, it is possible for most CNS neurons to form synapses. There are, however, some classes of neurons that rely on its effects for normal axonal development and elongation (Strittmatter *et al.*, 1995; Zhu and Julien, 1999).

A number of *in vivo* experiments have shown that the upregulation of GAP43 does not necessarily translate into successful regeneration of all adult CNS neurons. It is important to mention that the GAP43 gene promoter contains a binding site that is responsive to the AP-1 transcription factor, which as discussed bellow, is formed by the association of c-Jun and ATF3 (Weber and Skene, 1998). This means that the apparent upregulation of GAP43 could actually by the result of c-Jun upregulation (Hunt *et al.*, 2004). Placing a peripheral nerve graft into the thalamus of adult rats does lead to the upregulation of GAP43, followed by sprouting of thalamic neurons and regeneration of their axons into the graft (Vaudano *et al.*, 1995). This however does not occur in the case of cerebellar Purkinje neurons that do not upregulate GAP43 and fail to elongate their axons even in the presence of a peripheral nerve graft (Anderson *et al.*, 1998). When GAP43 is artificially overexpressed in Purkinje neurons, axonal sprouting does increase but this is still not sufficient to lead to regeneration into a peripheral nerve graft placed within the lesion site (Buffo *et al.*, 1997). Overall, these data suggest that the upregulation of GAP43 alone may not be a strong enough molecular stimulus to induce neurons that are intrinsically refractory to regeneration to elongate their axons even in the presence of a growth permissive environment like a peripheral nerve graft (Buffo *et al.*, 1997; Mason *et al.*, 2000).

c-Jun expression

c-Jun is a transcription factor which is primarily active during the embryonic stages of neuronal development (Herdegen *et al.*, 1993a; Herdegen *et al.*, 1993b; Herdegen *et al.*, 1997). Amongst its many functions, it has been shown to participate in the cell-cycle regulation, cellular differentiation and apoptosis in a variety of cell types (Angel and Karin, 1991; Jochum *et al.*, 2001). c-Jun forms heterodimers with proteins such as c-Fos or with ATF (Hai *et al.*, 1999). These

complexes comprise the AP-1 complex that goes on to initiate transcription from conserved TGACTCA sequences (Lee *et al.*, 1987; Angel *et al.*, 1988a) in response to stress signals such as cytokines and growth factors. The mechanism by which these factors go on to initiate the activation of AP-1 involve the N-terminal phosphorylation of c-Jun by the c-Jun N-terminal Kinases (JNKs) (Davis, 2000) which takes place at serine residues 62 and 73 and threonine residues 91 and 93. These residues are located within the c-Jun transactivating domain and their phosphorylation increases the transcription of a variety of genes including c-Jun itself (Angel *et al.*, 1988b). c-Jun is still expressed at low levels in the adult PNS as well as brain and spinal cord neurons (Herdegen *et al.*, 1997). Its expression is upregulated soon after an axotomising PNS lesion and high levels of expression are observed throughout the period necessary for target innervation to occur (Jenkins and Hunt, 1991). *In vitro* experiments have shown that this upregulation coincides with the onset of Wallerian degeneration and it is thought to be mediated by myelinating Schwann cells of the distal stump in response to losing contact with the degenerating axons. Moreover, c-Jun expression declines once re-myelination of regenerated axons occurs (Shy *et al.*, 1996). The involvement of c-Jun in neuronal regeneration has also been confirmed *in vivo* using conditional, c-Jun null mutant mice. After facial nerve axotomy these mice do not upregulate GAP43 while axonal sprouting and regeneration is significantly impaired compared to wild type mice (Raivich *et al.*, 2004). This elucidates that c-Jun plays an important role in the events following neuronal injury and axonal regeneration. Overall, the expression of c-Jun tends to be directly related to the ability of CNS neurons to initiate a regenerative response and extend their axons into a permissive environment such as a peripheral nerve graft. However, as was the case with GAP43, there appear to be some exceptions, which correlate with the intrinsic characteristics of particular groups of CNS neurons. For example, placing a peripheral nerve graft into the thalamus of adult rats can promote the regeneration of thalamic reticular nucleus neurons into the graft (Vaudano *et al.*, 1998; Chaisuksunt *et al.*, 2000). These neurons are able to express high levels of c-Jun after injury. In contrast, neurons of the substantia nigra are capable of regenerating their axons into a peripheral nerve graft but fail to upregulate c-Jun (Chaisuksunt *et al.*, 2003).

ATF3

ATF3 is a member of the ATF/CREB family of transcription factors. These have a highly conserved basic region leucine zipper (bZip) element that enables them to associate with other members of the same family of transcription factors (ATF/CREB) and form homodimers that suppresses expression from DNA containing the sequence TGACGTCA. Alternatively, ATF3 can form heterodimers by associating with c-Jun. These heterodimers form the AP-1 complex that by binding to the TGACTCA DNA sequence, it activates transcription (Hai *et al.*, 1999; Hai and Hartman, 2001). Following peripheral nerve axotomy, ATF3 is upregulated in both sensory and motor neurons of the spinal cord, in a similar manner to that seen in the case of c-Jun (Hai *et al.*, 1999). CNS intrinsic neurons that can successfully regenerate their axons into a peripheral nerve graft placed within the lesion site also appear to be able to upregulate the expression of ATF3. However, this appears to vary depending on the type of neuron and the distance of the neuronal cell body from the site of the injury. For example, corticospinal tract neurons are capable of upregulating ATF3 if the axotomy is carried out intracortically but fail to do so when the lesion is performed at the level of the spinal cord (Mason *et al.*, 2003).

Like the case of c-Jun, the expression of ATF3 is upregulated by Schwann cells in the distal stump of axotomised peripheral nerves. This increase in expression is initiated within 24 hours following injury, probably due to the fact that Schwann cells lose contact with the axons they used to myelinate (Hunt *et al.*, 2004). Expression of ATF3 declines once the axons have regenerated, but it can be switched on again if the regenerating axons are re-injured (Hunt *et al.*, 2004). In the same study it was shown that CNS glia present in the DREZ and in the dorsal columns do not upregulate the expression of ATF3 during Wallerian degeneration. This was different in the case of the optic nerve where ATF3 immunoreactivity was more evident within the lesion site but was comparatively weak in the distal or proximal stumps (Hunt *et al.*, 2004). Hunt and colleagues (2004) proposed that this may have been a reflection of the fact that CNS glia upregulate ATF3 in response to direct injury and not in response to Wallerian degeneration.

1.5 Neurotrophic factors and spinal cord regeneration

Neurotrophic factors are molecules whose biological activities involve cell survival, synaptogenesis and axonal plasticity. The term "neurotrophic factors" is used to describe the different families of molecules that share the ability to carry out these functions. These families include: the neurotrophin family, the neurokine family including the ciliary neurotrophic factor (CNTF), the TGF-related family including the glial-derived neurotrophic factor (GDNF) and the insulin-like growth factor family (IGF). Exogenous administration of neurotrophic factors has been proposed as a potentially therapeutic approach for inducing regeneration after spinal cord injury. The potential of this approach has been evaluated using various growth factors including BDNF (Houweling *et al.*, 1998; McTigue *et al.*, 1998; Jakeman *et al.*, 1998; Shumsky *et al.*, 2003; Tobias *et al.*, 2003; Zhou and Shine, 2003; Tobias *et al.*, 2005), bFGF (Lee *et al.*, 1999), NT3 (McTigue *et al.*, 1998; Shumsky *et al.*, 2003; Tobias *et al.*, 2003; Tuszynski *et al.*, 2003) and GDNF (Blesch and Tuszynski, 2003; Zhou and Shine, 2003). The following section focuses on the main characteristics of neurotrophic factors and their receptors with emphasis on the neurotrophin family. A more detailed account of their effectiveness in various CNS injury models is given in the relevant chapters of this thesis.

Neurotrophins were the first family of neurotrophic factors to be identified and its members include: NGF, BDNF, neurotrophin 3 (NT3), neurotrophin 4 (NT4) as well as the non mammalian neurotrophin 6 (NT6) and neurotrophin 7 (NT7) (Barde *et al.*, 1982; Leibrock *et al.*, 1989; Maisonpierre *et al.*, 1990a; Maisonpierre *et al.*, 1990b; Hallbook *et al.*, 1991; Ip *et al.*, 1992; Lai *et al.*, 1998). The neurotrophic factor NGF, was the first member of the neurotrophic family identified over 50 years ago (Levi-Montalcini, 1987). Its importance was revealed when sympathetic sensory ganglia of developing chicks treated with NGF *in vitro*, resulted in a characteristic halo of axonal outgrowth. During development, NT3, BDNF and NGF exhibit parallel as well as reciprocal patterns of expression (Maisonpierre *et al.*, 1990a). They are all upregulated between the 11^{th} and 12^{th} day of embryogenesis, which coincides with the onset of neurogenesis. However, the levels of expression for each neurotrophin vary

significantly, indicating that their roles are different. NT3 is predominantly expressed in spinal cord neurons and is associated with proliferation, migration and differentiation and its expression decreases as neurons reach maturation. In contrast, BDNF expression increases as CNS maturation progresses (Maisonpierre *et al.*, 1990a). NGF is expressed at relatively low levels in early spinal cord development during a period associated with naturally occurring death of newly formed motor neurons (Ernfors *et al.*, 1989). These differences correlate to a requirement for a separate physiological activity exerted by each neurotrophic factor at different stages of neuronal development. NT3 appears to be required for the early stages of development (Maisonpierre *et al.*, 1990b) and establishment of target innervation (Korsching and Thoenen, 1983), whereas BDNF acts as a maturation maintenance factor (Maisonpierre *et al.*, 1990a).

Apart from the NGF related neurotrophic factors there are two more classes of growth factors that have a neurotrophic function. Survival of motor and sensory neurons is supported by CNTF *in vivo* (Apfel *et al.*, 1993) while *in vitro* it increases survival and neurite outgrowth of purified embryonic motor neuron cultures (Arakawa *et al.*, 1990). GDNF deficient mice completely lack the enteric nervous system, including urethras and kidneys pointing to the potential role of this neurotrophin in organ development (Moore and Thanos, 1996). Neurotrophic factors have been the focus of extensive research in a variety of *in vivo* and *in vitro* CNS injury models [reviewed in detail for the corticospinal (Chapter 4), rubrospinal (Chapter 5) and optic nerve (Chapter 6) tracts]. The intracellular signalling events initiated by neurotrophic factors and their receptors are examined in the following section.

1.5.1 Neurotrophic factor receptors

Neurotrophic factors exert their effects by binding to their corresponding transmembrane receptors (Figure 1.5.1-a, page 45). Neurotrophic receptors can be divided in two classes: $p75^{NTR}$, a low affinity receptor that can bind every neurotrophin (Chao, 1994) and the Tyrosine Kinase (Trk) family of receptors, which display subspecies specificity for each neurotrophin (Barbacid, 1994).

Trk receptors

There are three distinct Trk receptors which have been characterised as transmembrane glycoproteins with a tyrosine kinase intracellular functional domain (Segal and Greenberg, 1996). NGF binds to TrkA (Kaplan *et al.*, 1991; Klein *et al.*, 1991a), BDNF to TrkB receptor (Klein *et al.*, 1991b) and NT3 to TrkC or TrkB (Lamballe *et al.*, 1991; Squinto *et al.*, 1991; Klein *et al.*, 1991b). Mutations in the TrkA gene have shown that it plays an important role in the development of nociceptive and thermoregulatory neuronal pathways (Smeyne *et al.*, 1994). In addition, studies utilising conditional knock-out TrkB mutants have revealed that this receptor is essential for synaptic plasticity and hippocampus mediated learning (Minichiello *et al.*, 1999; Tolwani *et al.*, 2002). Interestingly, BDNF and NT3 appear to have opposite effects in the myelination of the peripheral nervous system during development. NT3 inhibits myelination acting via its TrkC receptor while BDNF enhances myelination acting via the weak $p75^{NTR}$ receptor (Cosgaya *et al.*, 2002). NT3-mediated TrkC activation has been shown to enhance Schwann cell migration in the peripheral nervous system. This signalling pathway is mediated by the JNK cascade, regulated by the Rho GTPases Rac1 and Cdc42 (Yamauchi *et al.*, 2005). Upon binding a neurotrophic factor, the Trk receptor dimerises leading to the activation of the tyrosine kinase domain and consequent auto-phosphorylation of tyrosine residues. These are in turn recognised by specific signalling proteins, which share a *Src* homology domain, such as Phospholipase-C (PLC) and Phosphatidyl Inositol-3 Kinase (PI-3K) (Figure 1.5.1-a, page 45). Subsequently, second messenger proteins become activated, leading to multiple biological responses within the cell (Segal and Greenberg, 1996; Kaplan and Miller, 1997).

$p75^{NTR}$ receptor

$p75^{NTR}$ is a glycosylated transmembrane protein that belongs to the TNFα superfamily (Rao *et al.*, 1995) and exists in two isoforms: a full-length and a short-length form (Johnson *et al.*, 1986). Out of these, it is only the full-length version that is capable of neurotrophin binding, as the short form has only three of the four extracellular domains responsible for this function. $p75^{NTR}$ can modulate the response of a Trk receptor to its ligand. For example, $p75^{NTR}$ can

increase the affinity of TrkA for NGF so that the neuron concerned becomes responsive to lower concentrations of NGF (Hempstead *et al.*, 1991; Davies *et al.*, 1993). Interestingly, the precursor to the active form of NGF (pro-NGF) appears to bind to $p75^{NTR}$ with higher affinity than to TrkA. The reverse is true for the biologically active, mature NGF. It is thought that in the presence of pro-NGF, $p75^{NTR}$ acts as an apoptosis-inducing receptor, especially during neuronal development (Rabizadeh *et al.*, 1993). It does so via its own signalling pathway via the activation of the transcription factor nuclear factor (NF) κβ (Carter *et al.*, 1996). It is now thought that for this to occur, $p75^{NTR}$ has to interact with its co-receptor, Sortilin (Nykjaer *et al.*, 2004). This function can be silenced when the TrkA receptor is activated (Dobrowsky *et al.*, 1995). This $p75^{NTR}$-mediated apoptosis is thought to be behind the death of oligodendrocytes which occurs after a CNS axotomising lesion (Beattie *et al.*, 2002).

In addition to the above neurotrophin related functions, $p75^{NTR}$ can bind numerous other ligands, including the Nogo receptor (Wong *et al.*, 2002; Wang *et al.*, 2002b) and LINGO (Mi *et al.*, 2004). $p75^{NTF}$ signalling has been shown to increase Rho activation (Yamashita *et al.*, 1999) by interacting with the Rho GDP dissociation inhibitor (Rho-GDI). This leads to the subsequent activation of Rho GTPase and results in the inhibition of axonal growth (Yamashita and Tohyama, 2003). More importantly, there is evidence to suggest that this interaction between $p75^{NTR}$ and Rho-GDI is strengthened by the presence of Nogo or MAG (Yamashita *et al.*, 2002; Yamashita and Tohyama, 2003) which would fit in with their role as inhibitors to axonal elongation (discussed in later sections of this chapter).

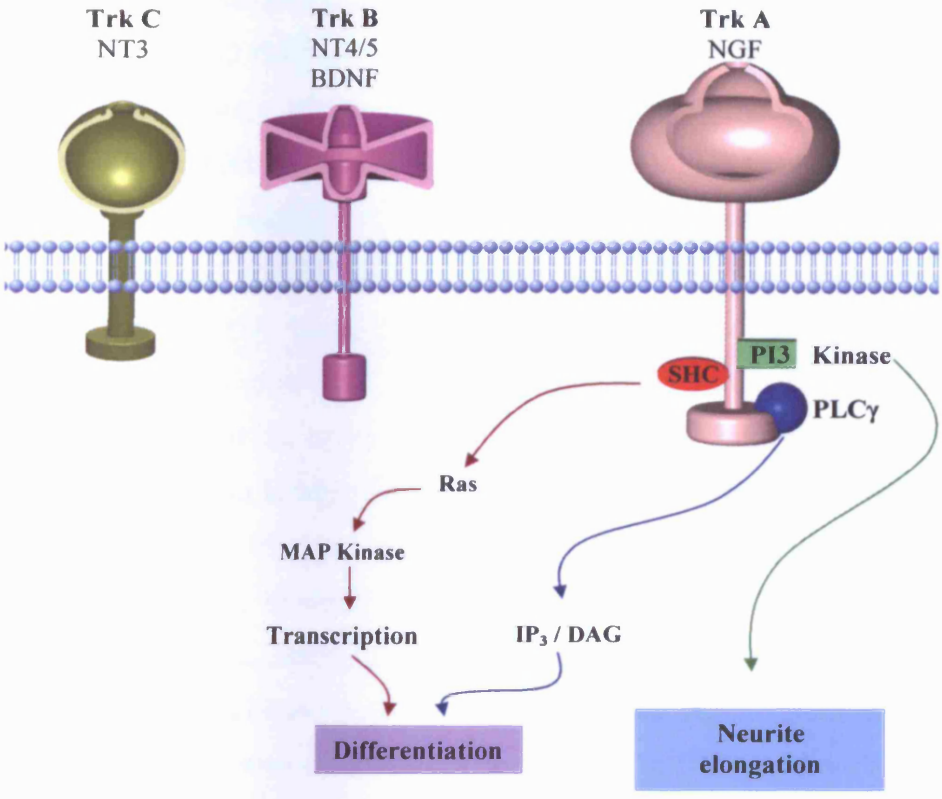


Figure 1.5.1-a: Neurotrophin receptors and their mediators.

1.6 Glial scar inhibitory molecules – Extrinsic factors

It has been hypothesised that reactive astrocytes may produce the required supportive molecules for axonal elongation but the corresponding receptors may be unresponsive at the site of injury. In contrast to the secretion of regeneration-supportive agents, reactive astrocytes also secrete molecules such as chondroitin sulphate proteoglycans (CSPG) such as aggrecan, brevican, neurocan, versican, phosphacan and NG2 (McKeon *et al.*, 1991). Structurally, these proteins have a large protein core, which has attached large and poly-sulphated glycosaminoglycan (GAG) (Morgenstern *et al.*, 2002). It is thought that it is this large GAG that is responsible for the inhibitory characteristics of CSPGs. Treatment with the enzyme chondroitinase ABC stops this inhibitory effect (Carulli *et al.*, 2005). However, the core proteins also contribute to this inhibitory function (Ughrin *et al.*, 2003). The manner in which CSPGs exert their inhibition is thought to involve the activation of a number of signalling pathways. This notion is based on the fact that neurons that come into contact with CSPGs appear to show enhanced activation of Rho-ROCK signalling pathway. In addition, blocking this pathway abolishes the CSPGs inhibitory effect (Monnier *et al.*, 2003). In experimental animals, blocking of Rho with protein kinase C inhibitors, promotes dorsal column regeneration after spinal cord injury (Sivasankaran *et al.*, 2004). In addition to CSPGs, there are several other molecules deposited at the lesion site that also hinder regeneration. For example, Semaphorin 3A (Sema3A) expression is upregulated by meningeal cells. Sema3A binds to neuropilin 1 which is also upregulated in injured CNS neurons. The interaction of Sema3A with neuropilin results in growth cone collapse (Sandvig *et al.*, 2004). Certain structural domains on semaphorins (thrombospondin repeats), physically interact with the long GAG chains on the abundant CSPGs and regulate their impact on CNS injured axons (Kantor *et al.*, 2004).

Tenascin-R is another cell adhesion protein that is upregulated following a CNS axotomising lesion and is also thought to exert a negative influence in the CNS axonal regeneration process (Gates *et al.*, 1996). Even though most of the cells around the lesion area that express tenascin also express GFAP, those located

within the lesion site are GFAP negative. This may suggest that tenascin expression was not only due to reactive astrocytes but also due to other cells attracted at the lesion site in response to injury: e.g. macrophages (Zhang *et al.*, 1997). Tenascin-R has been shown to inhibit axonal elongation (Probstmeier *et al.*, 2000). Tenascin-R knockout mice show increased recovery of function after spinal cord axotomy but this is thought to be due to the re-arrangement of spared fibres rather than due to frank regeneration of the injured neurons (Apostolova *et al.*, 2006).

Following axotomy, there is some unassisted sprouting of spinal cord neurons. This however ceases after a short period of time. Astrocytes have been shown to express a truncated form of neurotrophic receptor specific to neurotrophic factor BDNF (TrkB) which lacks its tyrosine kinase catalytic domain (Frisen *et al.*, 1993). *In vitro* experiments demonstrated that cells expressing this truncated form of the BDNF receptor were unable to support neurite outgrowth in the presence of exogenous BDNF. From those findings, Fryer (1997) hypothesised that neurotrophic factors may be available at the lesion site following injury but they are taken up by a truncated neurotrophin receptor on reactivated astrocytes of the glial scar, thus becoming quickly unavailable (Fryer *et al.*, 1997).

The role of the inflammatory response following an injury is controversial. Some studies suggest that the delayed recruitment of macrophages from the peripheral blood stream and the activation of resident microglia leads to the accumulation of debris such as myelin and axonal fragments (Perry *et al.*, 1993; George and Griffin, 1994). In addition, the ability of activated microglia to release cytotoxic factors at the site of injury has been suggested to have further side effects to the viability of neurons and their connections (Giulian *et al.*, 1994). However, when PNS neurons were transplanted in the CNS, regeneration was accompanied by mild inflammation at the PNS-CNS junction. This led to the hypothesis that low levels of inflammation may have positive effects in neuronal regeneration (Kliot *et al.*, 1990; Siegal *et al.*, 1990). The implication of axonal injury on the growth capacity of the affected neurons has also been considered as a causal agent in the regenerative failure. Neumann *et al.* (1999) have reported successful

regeneration of dorsal column fibres into and beyond the lesion site following adult spinal cord injury. In this experiment, the sciatic nerve of adult rats was transected one week before cutting the afferent sensory fibres of the ipsilateral dorsal root ganglion (Neumann and Woolf, 1999). It was hypothesised that the increased capacity of sensory neurons to regenerate is in part due to an increase in their growth state. Moreover, Filbin M. (1999) suggested that this regeneration, observed when a pre-conditioning lesions are inflicted, could be due to modified responses of the inhibitory molecules found in myelin and glial scar (Filbin, 1999). Her theory is based on the fact that the neuron's endogenous levels of cAMP dictate its response to inhibitors. It is thus likely that transection of dorsal root ganglia causes an increase in the intracellular levels of cAMP that resembles those found during developmental stages and leads to the neutralisation of inhibitory molecules (Chong *et al.*, 1996).

1.7 Myelin-derived inhibitors to CNS regeneration

CNS axons cannot regenerate following a mechanical lesion but can mount some regenerative response if the axotomising injury does not disturb any of the adjacent myelinated fibres (Berry, 1982). This observation led Berry and colleagues to propose that CNS myelin-contained elements were inhibitory to regeneration (For a review see: He and Koprivica, 2004).

Nogo

Confirmation that CNS myelin could directly inhibit neurite extension came later from a number of experiments that demonstrated that CNS but not PNS myelin was inhibitory to neurite extension (Savio and Schwab, 1989; Khan *et al.*, 1990). In addition, in co-cultures of adult neurons and dissociated oligodendrocytes, neurites were strongly repelled by these glial cells (Caroni *et al.*, 1988; Bandtlow *et al.*, 1990; Moorman, 1996). Size fractionation experiments of rat myelin constituents, revealed the presence of two protein: NI-35 and NI-250 which were also shown to be potent inhibitors of growing neurites *in vitro* (Caroni and Schwab, 1988). The monoclonal antibody IN-1, was shown to react with both these fractions, leading to increased neurite outgrowth both *in vitro* and *in vivo* (Caroni and Schwab, 1988; Schnell and Schwab, 1990; Schnell and Schwab,

1993; Bregman *et al.*, 1995). A partial sequence was eventually obtained for the NI-250 molecule which led to the cloning of the gene encoding Nogo (GrandPre *et al.*, 2000; Prinjha *et al.*, 2000). Blocking Nogo using IN-1 enhances sprouting of corticospinal axons, modestly improving their regeneration and reportedly enhancing functional recovery following spinal cord injury (Schnell and Schwab, 1990). In addition, it produces a modest increase in regeneration following an intracranial optic nerve injury (Weibel *et al.*, 1994). Nogo is a member of the reticulon family of transmembrane proteins and exists in three isoforms: A, B and C, thought to be produced via alternative splicing. NogoA has been shown to be highly expressed on the surface of oligodendrocytes (Huber *et al.*, 2002).

Myelin-associated glycoprotein (MAG)

MAG is an inhibitory molecule for the majority of adult CNS neurons (Mukhopadhyay *et al.*, 1994; DeBellard *et al.*, 1996) and is thought to be the main inhibitory component of myelin (McKerracher *et al.*, 1994). It is a transmembrane protein whose extracellular domain is expressed in the PNS and CNS (Lai *et al.*, 1987; Salzer *et al.*, 1987). Interestingly, MAG has two opposing functions depending on the age of the animal. For example, it was shown that MAG acts as a growth-promoting factor for axons during embryonic development and as an inhibitor after postnatal day 3 (Mukhopadhyay *et al.*, 1994; DeBellard *et al.*, 1996). Despite the fact that MAG is present in both the PNS and CNS myelin, it is only inhibitory in the CNS. It has been suggested that this may be due to the fact that PNS macrophages are able to remove the myelin debris very quickly after the mechanical damage (He and Koprivica, 2004). This is not the case following a CNS lesion (David *et al.*, 1990). Moreover, if MAG function is eliminated in chick E15 retina and optic nerve explants, then large numbers of RGC axons are able to regenerate across a crush site in the early myelinated optic nerve (Wong *et al.*, 2003).

Oligodendrocyte-myelin glycoprotein (OMgp)

OMgp is another putative inhibitor of neurite growth (Wang *et al.*, 2002a). It is a GPI-anchored protein that contains a leucine rich repeat (Mikol *et al.*, 1990). It has been shown to contribute to growth cone collapse *in vitro* (Kottis *et al.*,

2002) but its role in inhibiting CNS axonal elongation *in vivo* is still unclear. OMgp is expressed on oligodendrocytes and on the myelin sheaths that are in the immediate vicinity of CNS axons. The onset of its expression appears to coincide with the initiation of myelination (Mikol and Stefansson, 1988; Mikol *et al.*, 1993; Habib *et al.*, 1998). In particular, it was recently shown that it is prominently expressed near the nodes of Ranvier and may have a role in preventing collateral sprouting (Huang *et al.*, 2005). Recombinant OMgp, was shown to induce growth cone collapse in cultured neurons (Wang *et al.*, 2002a; Barton *et al.*, 2003).

Down-stream effectors

All of these molecules have recently been shown to exert the majority of their inhibitory influence by binding to the Nogo receptor (NgR) (Fournier *et al.*, 2001; Fournier *et al.*, 2002; Hunt *et al.*, 2002b; McGee and Strittmatter, 2003). MAG and OMgp have also been shown to act through the cAMP/pKA/Arginase pathway, independently from NgR (Cai *et al.*, 2002). All these molecules have a common characteristic: they all signal via the small Rho GTPase signalling pathway. RhoA belongs to a family of GTPases that regulate cytoskeletal dynamics of growth cones and neurite extension. It has been shown that a dominant negative RhoA can enhance RGC axonal regeneration *in vitro*. This effect is also evident *in vivo* as treatment with C3, a RhoA inactivator, was shown to induce regeneration in the crushed optic nerve (Lehmann *et al.*, 1999).

1.8 Strategies for inducing regeneration in the injured CNS

Spinal cord injury poses a multifactorial problem and as such it requires a combination of different treatments. Over the last decade research has focused on combining different treatments for promoting neuronal survival and regeneration in a variety of different paradigms. Such reports are reviewed and discussed in relation to the corticospinal (Chapter 4), rubrospinal (Chapter 5) and optic nerve (Chapter 6) tracts and they are briefly summarised here.

1.8.1 Cellular interventions

This technique aims to provide a "bridge" that links the proximal and distal stumps, normally separated by an acellular cyst, whose presence hinders any regenerative attempt. This can be achieved by placing a growth-promoting substrate into the lesion site. Such substrates can be peripheral nerve grafts, Schwann cells or olfactory ensheathing glial (OEG) cells (for a review see Thuret *et al.*, 2006).

Peripheral Nerve Grafts

The rationale behind the use of peripheral nerve grafts has been discussed in previous sections, while the application of this technique in rodent models of spinal cord and optic nerve injury is further discussed in detail in the relevant Chapters (4-6). Peripheral nerve grafts have been used either alone or in combinations with modulators of the inflammatory response or neurotrophic factors such as acidic FGF and fibrin glue (Lee *et al.*, 2002; Cheng *et al.*, 2004; Lee *et al.*, 2004). The combination of acidic FGF and a peripheral nerve graft has been used in a human case of a chronic paraplegia (Cheng *et al.*, 2004). In this isolated case the authors report some recovery of function but this was not replicated in a case of complete spinal cord transection (Amador and Guest, 2005).

Schwann Cell Grafts

A considerable number of studies document that these cells have the ability to induce axotomised CNS neurons to regenerate. When these cells were placed as a suspension into the lesion site of a transected spinal cord, they induced sensory and motor neurons to extend their axons into the graft (Takami *et al.*, 2002). In addition, the authors report that this is accompanied by some recovery of function (Takami *et al.*, 2002). In another experiment, Schwann cells embedded within channels containing extracellular matrix, were transplanted into the site of a lateral cord hemisection. These animals also received a combination of BDNF and NT3 (Xu *et al.*, 1995; Bamber *et al.*, 2001). Injured axons could again be seen entering the graft but as in the previous experiment by Takami *et al.* (2002), they failed to enter the distal stump (Xu *et al.*, 1995; Pinzon *et al.*, 2001).

Olfactory Ensheathing Glial Cells

OEGCs have been the focus of considerable research in CNS regeneration paradigms and the injured spinal cord in particular. This type of glial cell is responsible for ensheathing the axons of olfactory receptors which are also of neuronal origin. These neurons are unique in that they can regenerate their axons through the adult CNS environment. The potential of these cells to influence axonal regeneration was elucidated when purified cultures where transplanted into the dorsal horn of a spinal cord that had previously received a rhizotomy (Ramon-Cueto *et al.*, 1993). The authors found that the lesioned DRG axons were able to regenerate into the ipsilateral dorsal horn. Despite their original promise, OEGCs have not been conclusively shown to promote regeneration in the injured spinal cord with often contradicting reports regarding their effectiveness. Some groups report significant regeneration through the CNS environment (Raisman, 2001). Recovery of function and axonal regeneration has been reported in paradigms where OEGCs were transplanted into the lesion site, either immediately or within 2 months following spinal cord axotomy (Lu *et al.*, 2001; Keyvan-Fouladi *et al.*, 2003; Li *et al.*, 2004; Li *et al.*, 2005; Collazos-Castro *et al.*, 2005). Other groups report that OEGCs fail to promote any regeneration in similar spinal cord injury models (Gomez *et al.*, 2003; Riddell *et al.*, 2004). In addition, another group has found that OEGCs are not as efficient at promoting regeneration as Schwann cells in the injured rat spinal cord (Takami *et al.*, 2002). Surprisingly, and despite the uncertainty, transplants of cells derived from foetal or adult olfactory bulbs have been grafted into humans in clinical research centres in China, Portugal and Colombia (Huang *et al.*, 2003; Dobkin *et al.*, 2006) (also reviewed in Thuret *et al.*, 2006).

Counteracting myelin inhibitors

Numerous strategies have been employed in order to counteract the effects of myelin-derived inhibitors to axonal regeneration. Their effect on axotomised CNS neurons has been assessed in a variety of experimental paradigms (Sandvig *et al.*, 2004; Yiu and He, 2006). These studies and their main findings are further discussed in the relevant chapters of this thesis.

1.8.2 Gene Therapy for inducing CNS regeneration

A plethora of *in vivo* and *ex-vivo* genetic engineering techniques have been developed with the aim of inducing and supporting the generation of injured CNS neurons. As previously discussed, the CNS response to injury is a complex mechanism involving neurons themselves and their extracellular environment. It therefore follows that for a regenerative approach to be successful it must augment all stages of CNS regeneration. Potentially therapeutic genes and factors that can counteract inhibitory cues must be provided over three time windows: As soon as injury is inflicted (i.e. emergency damage control) aiming to rescue injured neurons and prevent inhibition from the forming glial scar. It must be able to sustain transgene expression for a significantly long period of time in order to support and direct neurite outgrowth from sprouting axons. It must also be able to supply factors that can aid in the recognition of target tissue and facilitate the formation of synapses (Breakefield *et al.*, 1999).

The appeal of utilising gene therapy vectors for promoting regeneration is based on the fact that they enable the cell bodies of injured CNS neurons themselves to synthesise the growth promoting factors they need in order to enhance their limited regenerative potential. This means that the factors produced are not subject to the usual limitations of accessing CNS neurons, imposed by the protective blood-brain barrier. In addition, molecules such as neurotrophins have an extremely short half-life and quickly become degraded. This is an important impediment as for regeneration to occur such growth promoting factors must be present for a considerable length of time. Alternative methods, such as osmotic min-pumps also aim to ensure prolonged presence of trophic molecules but have the added disadvantage that they further compromise local tissue integrity. Furthermore, neurotrophic factors have a number of functions in addition to their role in axonal growth. They are anterogradely transported and released to post-synaptic targets. By limiting their production to the small number of neurons affected by the lesion helps to ensure that the effects they exert remain local thus minimising their potential for non-specific, side effects. Finally, in contrast to the use of *ex vivo* approaches, gene therapy techniques such as viral vectors can be delivered via minimally invasive, stereotactic procedures that do not exacerbate a

pre-existing injury. Considerable research has led to the production of a number of vector systems that can be used in the injured CNS. These range from non-viral systems such as liposomes, to viral vector systems based on the use of Adenovirus (AV), Adeno-associated virus (AAV), lentivirus and retrovirus backbones (Breakefield *et al.*, 1999; Abdellatif *et al.*, 2006). A number of studies have emerged that report variable degrees of success with these systems within the context of inducing regeneration of injured spinal cord neurons. These are reviewed and discussed in the relevant Chapters of this thesis.

1.8.2.1 Herpes simplex virus

The innate characteristics of HSV1 have long been exploited in order to produce a gene delivery tool for a variety of applications. In particular, the innate characteristics of this virus have pointed towards its potential as a powerful tool for gene delivery applications to the nervous system (reviewed in Lilley and Coffin 2003). Firstly, it has a natural propensity to infect neuronal cells where it can establish life-long latency. Secondly, it does not integrate into the host genome but elements of its latency can be exploited to confer long-term foreign gene expression. Thirdly, the genome of HSV1 is capable of accepting large inserts such as foreign genes but also other DNA based elements such as expression cassettes that can maximise gene expression. The features summarised above are the basis of utilising this virus for delivering regeneration promoting genes to the injured spinal cord, under consideration in this study.

1.9 Biology of HSV1

HSV is a member of the alpha *herpesviridae* family of double stranded DNA (dsDNA) viruses and exists in two subtypes: HSV1 and HSV2 (Roizman *et al.*, 1981). These two viruses have different clinical manifestations. HSV2 causes genital warts while HSV1 causes recurrent mucocutaneous lesions (cold sores) in only one third of the population (Roizman and Sears, 1996) despite it being seroprevelent in >80% in some populations (Sharp, 2002). Very rarely and primarily in immunocompromised hosts, wild type virus can enter the CNS and cause viral encephalitis. In order to understand the steps taken for the production

of non-toxic HSV1 based vectors suitable for delivery to the CNS (described in Chapter 3) it is important to have an understanding of the life cycle of the wild type virus. The salient features of the physiology of HSV1 and gene expression patterns noted in the lytic and latent phases of its life cycle are reviewed in the sections that follow.

1.9.1 Structure of the virion

The mature HSV1 virion can be divided into four morphologically distinct structural layers: the double stranded viral DNA core, the protein capsid, the proteinaceous tegument and finally the glycoprotein containing viral envelope (Steven and Spear, 1997). A schematic representation of an HSV1 virion is given in Figure 1.9.2-a (panel **A**, page 58). The 152kb double stranded viral DNA genome encodes 84 viral proteins and is encased in the protein capsid. The capsid has an icosahedral morphology, consisting of 161 capsomers (Steven and Spear, 1997; Kasamatsu and Nakanishi, 1998). The proteins involved in the formation of this icosahedron are: VP5, VP19, VP23 and VP26 (Zhou *et al.*, 1995; Bowman *et al.*, 2003). The nucleocapsid, measures 125nm in diameter (~1250Å) and is surrounded by the amorphous tegument (Zhou *et al.*, 1994; Zhou *et al.*, 2000; Baker *et al.*, 2003). The tegument constitutes ~65% of the total virion mass, even though it only contains 14 different viral proteins. These include proteins such as VP16 (also known as α-TIF, ICP25, vmw65 and UL48), involved in viral gene transcription (Stern *et al.*, 1989; Wysocka and Herr, 2003), the virion host shut-off protein (vhs or UL41) responsible for the suppression of host cell protein synthesis (Kwong and Frenkel, 1989) and a number of glycoproteins (g) involved in viral egress from the infected cell (Granzow *et al.*, 2001; Foster *et al.*, 2003). The bi-layered external envelope, bearing short protein spikes, surrounds the tegument and nucleocapsid giving the enveloped virion a mean diameter of 200 – 300 nm (~2000Å) (Whitley, 1996; Steven and Spear, 1997). Overall the envelope contains 12 proteins, which correspond to the protein spikes protruding from the lipid envelope. Ten of these proteins correspond to viral glycoproteins that play a role in virus host immune evasion (Lubinski *et al.*, 2002; Friedman, 2003), attachment, host cell entry (Spear, 2004) and cell-to-cell propagation (Dingwell *et al.*, 1994).

1.9.2 Virion attachment and host cell entry

HSV1 enters host cells via a pH-independent mechanism that occurs in two stages: virion attachment to the host cell and subsequent fusion of the viral envelope with the plasma membrane (reviewed in Spear 2004 and Frampton *et al.* 2005). These two processes involve a series of poorly understood interactions between virion glycoproteins and various host cell receptors. Only four envelope glycoproteins have been shown to be essential for HSV1 infection, at least *in vitro*: gB, gD, gH and gL (Desai *et al.*, 1988; Cai *et al.*, 1988; Shieh *et al.*, 1992; Hutchinson *et al.*, 1992; Fuller and Lee, 1992). Deletion mutants for one or more of these glycoproteins results in the production of enveloped virions that are still able to attach to the host cell membrane but cannot penetrate the cell membrane as they fail to initiate the entry cascade (Cai *et al.*, 1988; Ligas and Johnson, 1988; Fuller *et al.*, 1989; Forrester *et al.*, 1992; Roop *et al.*, 1993). On the contrary, gC and gC/gB deletion mutants can penetrate the cell membrane but the efficiency of initial virion attachment to the target cell plasma membrane is reduced 10-fold (Svennerholm *et al.*, 1991; Laquerre *et al.*, 1998). These experiments point to the fact that attachment and entry are two separate mechanisms that rely on separate glycoproteins. Attachment is thought to involve interactions between the positively charged sequences on virion glycoproteins gC (Tal-Singer *et al.*, 1995) and gB (Herold *et al.*, 1995) with the negatively charged elements of heparan sulphate moieties found on cell surface proteoglycans (WuDunn and Spear, 1989; Shieh *et al.*, 1992; Laquerre *et al.*, 1998). This is supported by the findings that heparin can inhibit virion attachment (Herold *et al.*, 1995) while cell lines that are deficient in the synthesis of heparan sulphate are 85% resistant to HSV1 infection (Shieh *et al.*, 1992). In addition, the simultaneous deletion of gC and the heparan sulphate binding domain of gB results in a reduction in the efficiency with which the virion binds to the target cell membrane. This reduction in efficiency is similar to that seen in the previously mentioned heparan sulphate deficient cell lines (Gruenheid *et al.*, 1993). It becomes evident therefore that gC and gB are the key glycoproteins involved in this first stage of virus infection. Interestingly, gC is also thought to block the complement-mediated neutralisation of the virus by binding to the C3b element of the complement cascade (Fries *et al.*, 1986; McNearney *et al.*, 1987).

Following attachment, the virus becomes internalised into the host cell. The attachment of the virion facilitates the subsequent binding of gD to specific receptors on the target cell surface. The instrumental role gD has in the ability of the virus to enter the host cell is shown by the fact that pre-incubating host cells with soluble gD prior to infection, prevent infection by wild type HSV1 (Dean *et al.*, 1994). Overall, three receptors have been identified as targets for gD (Figure 1.9.2-a, panel **B**, page 58): Firstly, the herpes virus entry mediator (HVEM or HveA), a member of the TNFα/NGF receptor family. HVEM was identified by screening of a cDNA expression library for clones that enabled virus infections in a CHO cell line that was resistant to HSV1 infection (Montgomery *et al.*, 1996). HVEM is expressed in a variety of cell types including lymphocytes, fibroblasts and epithelial cells and it is thought that HVEM signalling may be involved in the regulation of the host immune response (Kwon *et al.*, 2003). Secondly, Nectin-1 (HveC) (Geraghty *et al.*, 1998), a member of the Nectin group of adhesion molecules belonging to the immunoglobulin superfamily. Nectin-1 is involved in cell-cell adhesion (Takahashi *et al.*, 1999; Sakisaka *et al.*, 2001) and synapse formation (Mizoguchi *et al.*, 2002) and is thought to be the main receptor for HSV1 entry in peripheral sensory neurons (Richart *et al.*, 2003). Nectin-1 mRNA is found in abundance in the human CNS (Cocchi *et al.*, 1998), in neuronal cell lines (Geraghty *et al.*, 1998), peripheral sensory neurons, sympathetic and parasympathetic ganglia (Haarr *et al.*, 2001). Thirdly, 3-*O*-sulphated heparan sulphate, an alternative version of the heparan sulphate moiety, generated by 3-*O*-sulfotransferases (Shukla *et al.*, 1999). It is generally believed that the binding of gD to its cellular receptors serves two functions. Firstly it stabilises the virion-host cell attachment initiated via the gC/gB binding on cell surface heparan sulphate and secondly it serves to initiate the fusion of the viral envelope and the host cell plasma membrane. The exact sequence of events that lead to fusion and virion entry is not fully understood. One hypothesis is that the binding of gD to one of its receptors results in a conformational change in this glycoprotein that enables it to interact with gB and/or gH/gL (Fuller and Lee, 1992). Alternatively, the binding of gD enables gB and gH/gL to bind to their own receptors, that can initiate their fusogenic activity without the direct participation of gD (Spear and Longnecker, 2003).

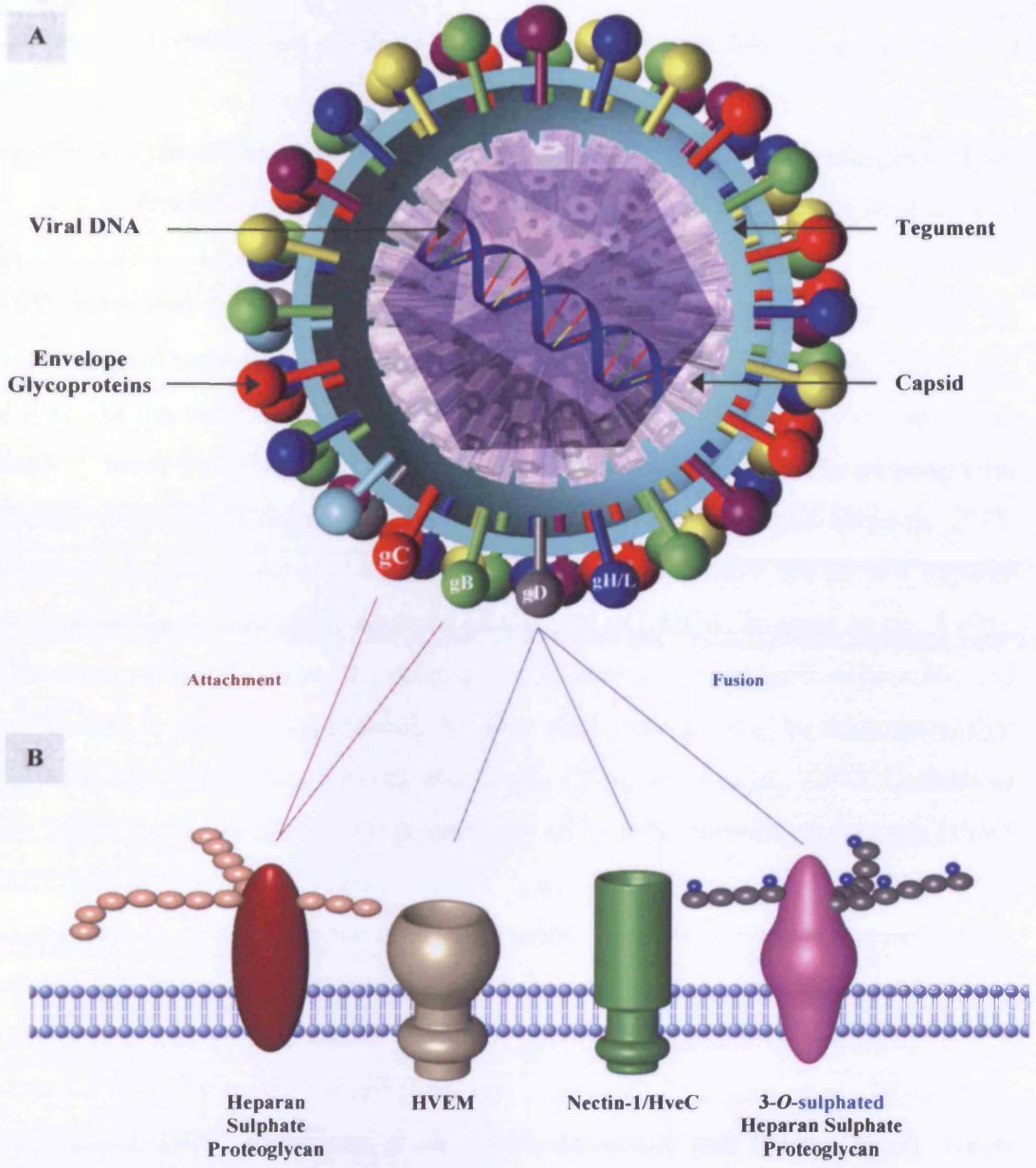


Figure 1.9.2-a: Structure of the HSV1 virion and viral entry.

A. The virion structure is based on the theoretical mathematical parameters of an icosahedral structure[2]. The capsid is composed of 12 vertices and 20 facets, each of which is an equilateral triangle and displays an overall 5:3:2 symmetry. This results from: six 5-fold axes of symmetry passing through the vertices, ten 3-fold axes passing through each face and fifteen 2-fold axes passing through each of the sides of the icosahedron (Caspar and Klug, 1962).

B. Attachment of the virion involves the binding of gC and gB to heparan sulphate moieties on proteoglycan chains (depicted in red). This interaction facilitates the binding of gD to: *1.*HVEM, a member of the TNF/NGF receptor family (gray), *2.* the Nectin-1 receptor a member of the immunoglobulin superfamily (Nectin-1/HveC, green) and *3.* 3-O-sulphated Heparan Sulphate proteoglycan (pink with sulphate groups shown in blue). Binding of gD with the involvement of gB and gH/L, triggers the fusion of the envelope and subsequent release of the nucleocapsid into the host.

[2] Büchen-Osmond, C. (Ed) (2004). 00.031.1. Alphaherpesvirinae. In: *ICTVdB - The Universal Virus Database*, V3. ICTVdB Management, Columbia University, New York, USA, http://www.ncbi.nlm.nih.gov/ICTVdb

1.9.3 Trafficking of HSV1 in the nervous system following infection

Upon coming into contact with a potential animal host, HSV1 initially infects epithelial cells of the skin and mucosal surfaces. The virus then undergoes a lytic cycle and replicates at the site of primary infection, destroying the host cells in the process. Clinically this manifests as *herpes labialis*, a blistering mucocutaneous lesion (cold sore) which serves the purpose of disseminating the virus and aid transmission to another host (Roizman and Sears, 1996; Flint *et al.*, 2004). As the local number of particles increases, the virus enters the underlying sensory nerve terminals and is retrogradely transported to the nuclei of peripheral sensory neurons in dorsal root or trigeminal ganglia (Cook and Stevens, 1973; Topp *et al.*, 1994; Bearer *et al.*, 2000). For the next few days, the virus is capable of replicating within these neurons (Knotts *et al.*, 1974; Kramer *et al.*, 1998). However, within ~7 days, the virus enters latency and viral gene expression and replication is quickly suppressed. No free viral particles can be detected within neurons after that time (Stevens and Cook, 1971; Walz *et al.*, 1974; Cabrera *et al.*, 1980; Fraser *et al.*, 1981). A summary of events following a primary HSV1 infection is shown in Figure 1.9.4-a (page 63). During latency, lytic gene expression is silenced as the double stranded viral DNA becomes quiescent. It adopts a circular, episomal structure that becomes associated with nucleosomes (Deshmane and Fraser, 1989). The viral genome is capable of persisting in this state for the lifetime of the cell (Nesburn *et al.*, 1972; Stevens *et al.*, 1972; Rock and Fraser, 1983; Efstathiou *et al.*, 1986; Mellerick and Fraser, 1987). Upon appropriate stimuli such as stress, trauma or drug induced immuno-suppression (Latchman, 1990), the virus may periodically reactivate and re-enter the lytic cycle. Latent virus reactivation occurs in only a small proportion of latently infected neurons (Baringer and Swoveland, 1973) and is the mechanism responsible for the recurrent nature of HSV1 infections (Baringer and Swoveland, 1973; Knotts *et al.*, 1974). After reactivation, the resulting viral progeny can follow two routes: either be transported anterogradely back to the primary site of infection where secondary lesions develop (Roizman and Sears, 1996) or be retrogradely transported to the CNS where if lytic expression is established, it will result in either aseptic meningitis or encephalitis (Knotts *et al.*, 1974; Enquist *et al.*, 1998; Roizman and Whitley, 2001).

HSV1 encephalitis is a rare complication, with an incidence of 1:250,000 population/year in the USA and 2.5 per 1 million of population in Sweden (Skoldenberg *et al.*, 1984; Whitley and Roizman, 2001). Even though rare, if left untreated herpes encephalitis has a mortality of >70% (Tyler, 2004). The exact pathway via which the virus reaches the CNS in human hosts in order to cause encephalitis is unclear. One hypothesis is that latent virus reactivating in trigeminal or sensory ganglia, instead of spreading via its usual anterograde route to cause secondary epidermal infections may spread via tentorial nerves to the anterior and medial cranial fossa (Davis and Johnson, 1979). Alternatively, and to explain the occurrence of HSV1 encephalitis following a primary infection, virus could be retrogradely transported via olfactory nerve pathways to the orbitofrontal and medial temporal lobes (Tyler, 2004).

1.9.4 Intra-axonal transport mechanisms

The use of the terms *anterograde* and *retrograde* can be confusing as they are used in the literature to describe both the movement of the virus between first and second order neurons and intra-axonal transport. So, when describing aspects of the HSV1 life cycle, such as lytic events or reactivation, the terms are used to describe the directional crossing of synapses (Enquist *et al.*, 1998). The same terms are also used to describe the mechanisms involved in the transport of the virion within a single neuron. In this case, retrograde transport describes the movement of the virion from axonal terminals to the cell nucleus and anterograde the reverse. It has been estimated that it would take an average of 231 years for a herpes virus capsid to diffuse 10mm in the axonal cytoplasm (Sodeik, 2000). In reality, this is an active process which is reflected in the fact that actual rate of transport for HSV1 nucleocapsids *in vivo* is 0.3-3.5 μm/s retrogradely (Smith *et al.*, 2001) and 0.6 μm/s anterogradely (Penfold *et al.*, 1994). Microtubule-inhibiting drugs such as colchicine, nocodazole and vinblastine inhibit HSV1 axonal transport, demonstrating that the virus utilises this intra-axonal microtubule system (Topp *et al.*, 1994; Sodeik *et al.*, 1997). The interactions between the nucleocapsid and tegument components and elements of the microtubule mechanism are the focus of this section (schematically represented in Figure 1.9.4-b, page 63 and reviewed in Frampton *et al.* 2005).

The microtubule system is capable of bidirectional movement of intracellular materials due to its plus and minus polarity, with the plus ends pointing towards the axon terminals and the minus ends pointing towards the nucleus. Retrograde transport occurs in a plus to minus orientation and takes place following primary infection of sensory neurons as the nucleocapsid and tegument are transported from neuronal terminals, along the axon to the cell body and finally the nucleus (Frampton, Jr. *et al.*, 2005).

Cytoplasmic dynein is the only motor protein responsible for retrograde transport in non-dividing cells such as neurons (Paschal and Vallee, 1987). It is a large protein complex (1.2MDa) composed of heavy and light chains. Heavy chains carry out the motor function, while the intermediate and light chains interact with the loading cargo (King, 2000). Immunolabelling studies have shown that HSV1 capsids colocalise with cellular dynein (Dohner *et al.*, 2002). The HSV1 tegument protein UL34, has been shown to specifically interact with the neuronal isoform of the dynein intermediate chain 1a (IC-1a) (Ye *et al.*, 2000). It is however possible that the UL34 protein may not be the sole component of this virion-dynein interaction as it is absent from mature virions and it is therefore doubtful if it would be present during infection (Reynolds *et al.*, 2002; Douglas *et al.*, 2004). Douglas *et al.* (2004) recently suggested that virion protein 26 (VP26), a capsid component, also interacts with the light chains of dynein during infection. These authors suggest that VP26 may be more suited to this role than UL34 because unlike UL34, VP26 is a component of the capsid and not the tegument, which is quickly lost after cell entry (Wingfield *et al.*, 1997). Other tegument or virion proteins that have been suggested to play a perhaps less important role are VP1/2 and UL25 (Tomishima *et al.*, 2001; Dohner *et al.*, 2002). However, further research is needed into their role, as currently the extent and nature of their involvement is unclear. During reactivation from latency, virion progeny is transported in a minus to plus orientation (Frampton, Jr. *et al.*, 2005). Virions are propelled anterogradely down the axon towards neuronal terminals in a process mediated by kinesins, a family of microtubule-associated motor proteins. Kinesins exist in two isoforms: ubiquitous and neuronal (Hirokawa *et al.*, 1991; Niclas *et al.*, 1994; Goldstein and Yang, 2000).

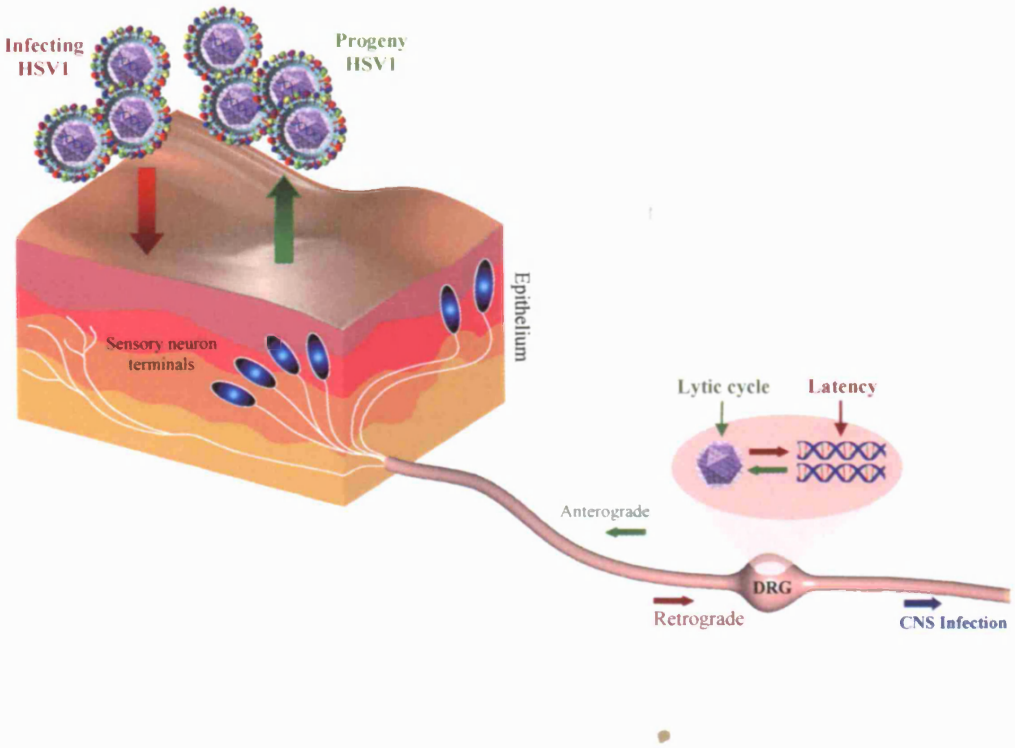


Figure 1.9.4-a: Neuronal trafficking of HSV1*in vivo*.

Following infection and replication within the epithelial cells of mucosal surfaces, virions are taken up by sensory neuron terminals and are retrogradely transported into the nuclei of peripheral DRG or trigeminal neurons where they can establish latency. The dsDNA genome can remain in this quiescent state for the lifetime of the host. When conditions are favourable the latent genome can reactivate and enter a lytic cycle. Progeny virions are then anterogradely transported to the neuronal terminals where they manifest as herpes labialis (cold sores). Alternatively, virions can enter the CNS where they can again either enter latency or cause lethal encephalitis.

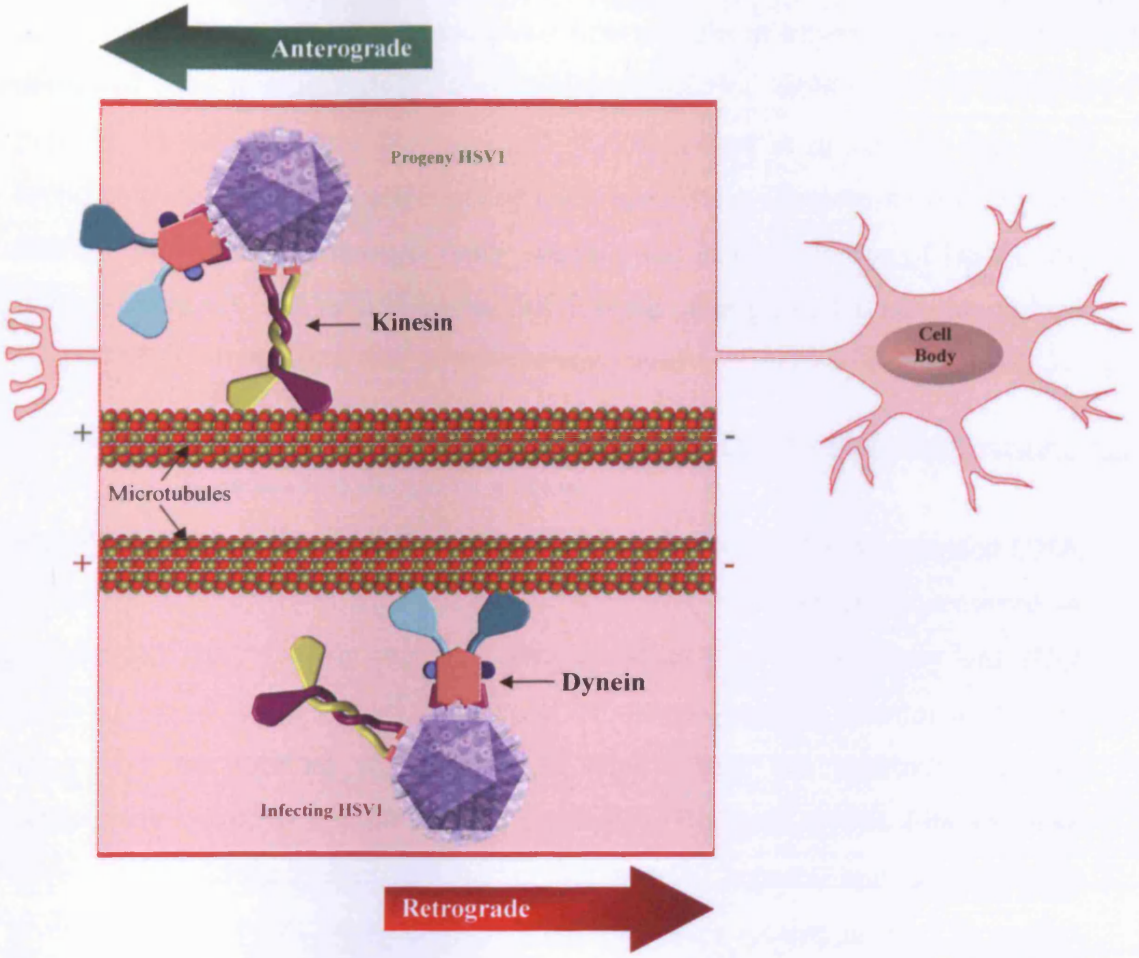


Figure 1.9.4-b: Anterograde and retrograde mechanisms of HSV1 axonal transport.

Microtubules are hollow tubulin cylinders with a negative and a positive end attached to the centrosome (Nogales, 2000). Incoming nucleocapsids are actively transported from neuronal terminals towards the host nucleus (retrogradely) in a mechanism that makes use of the +/- polarity of the axon microtubule system. Infecting HSV1 capsids attach to dynein via the viral protein VP26 (UL35) and is retrogradely transported to the nucleus in a plus to minus direction (Dohner *et al.*, 2002; Douglas *et al.*, 2004). Progeny procapsids are transported from the cell body towards neuronal terminals (anterograde). The viral tegument protein US11 directly interacts with Kinesins (Diefenbach *et al.*, 2002), a group of microtubule associated motor proteins that carry pro-capsids in a minus to plus direction (Flint *et al.*, 2004).

Interestingly, a number of reports suggest that nucleocapsids and tegument travel separately from envelope components such as virion glycoproteins (Miranda-Saksena *et al.*, 2002; Frampton, Jr. *et al.*, 2005). The HSV1 tegument component US11, a viral RNA-binding protein (Roller and Roizman, 1992), has been shown to associate directly with the ubiquitous heavy chain of kinesin (Diefenbach *et al.*, 2002). In a recent study, the amyloid precursor protein (APP), itself a putative kinesin receptor (Kamal *et al.*, 2000; Kamal *et al.*, 2001), has been found to be an abundant component of the herpes virion (Satpute-Krishnan *et al.*, 2003). The authors of the later study suggest that in the presence of HSV1, the normal transport of APP is disrupted and it is this disruption that may precipitate the pathology associated with Alzheimer's disease.

1.9.5 Structure of the HSV1 genome

The HSV1 genome is a linear, 152Kb (Kieff *et al.*, 1971), double stranded DNA molecule (reviewed in Roizman and Sears 1996). Viral genes are arranged in segments (Figure 1.9.5-a, page 65): a unique short (U_S) and a unique long (U_L) region, each of which is flanked by a pair of inverted repeats: a terminal (TR_L or TR_S) and an internal repeat (IR_L or IRs). There are approximately 84 polypeptides expressed from the HSV1 genome (Roizman, 1996). Five of those have open reading frames (ORFs) in the inverted repeats, and are therefore present in two copies per genome. In addition, ORFs located in the TR_L region are responsible for a mixed population of RNA transcripts produced during latency, termed latency associated transcripts (LATs), which will be discussed in section 1.9.7 (page 75). These ORFs lead to the production of a variety of viral proteins some of which are essential for viral replication *in vitro* (termed essential) and proteins whose function is not a prerequisite for viral propagation *in vitro* (termed non-essential). Viral genes fit into three categories, based on the timing of their expression during replication. Thus, the first viral proteins to be transcribed are the Immediate Early (designated IE or α) proteins, followed by the Early (E or β) group of proteins and finally the Late (L or γ1/γ2) genes. Their expression is tightly regulated and occurs in a cascade manner. A schematic representation of the sequential nature of IE, E and L gene transcription is shown in Figure 1.9.6-a (page 70, panel **B**).

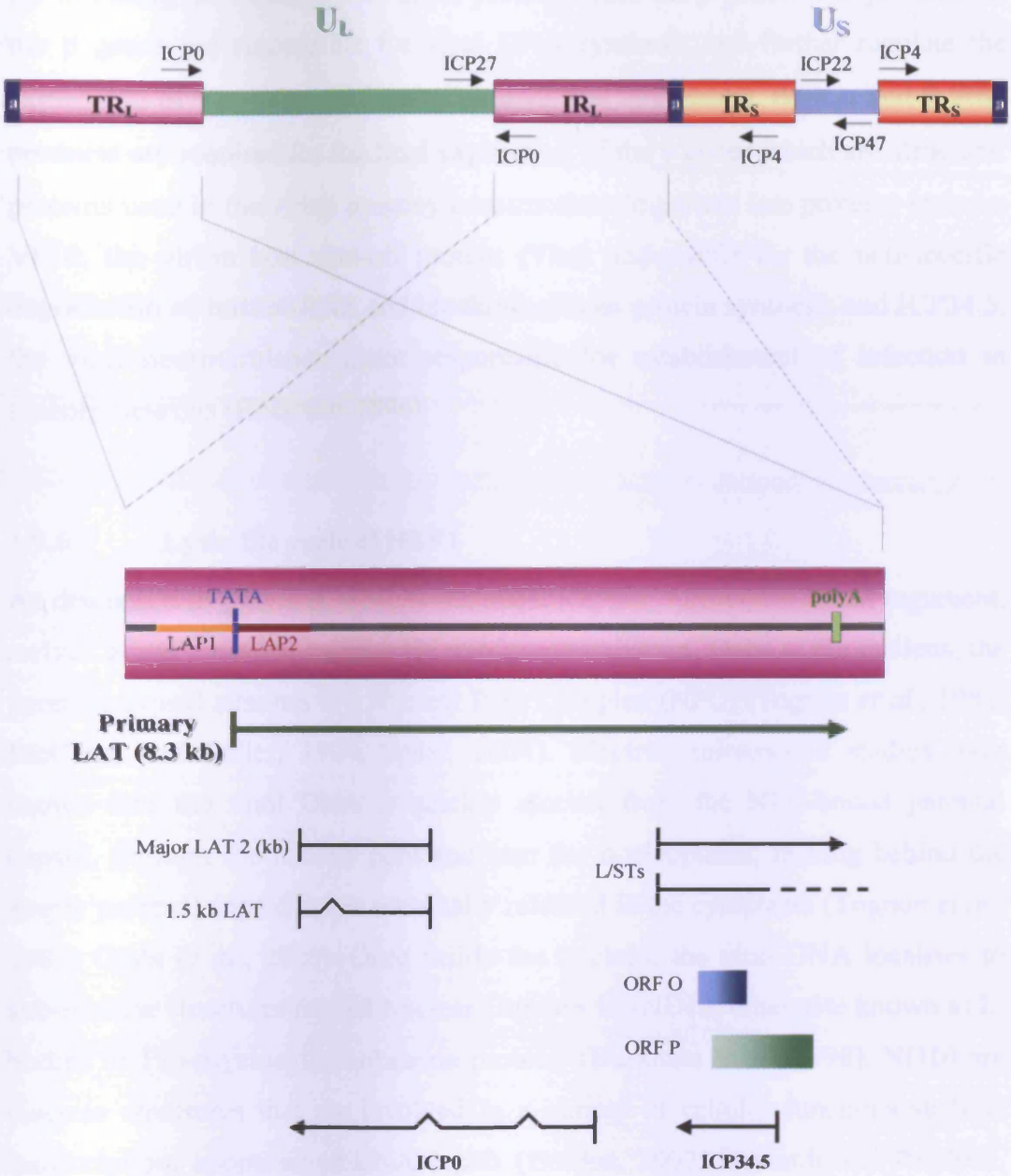


Figure 1.9.5-a: Structure of the HSV1 genome & map of the LAT transcriptional unit.

The HSV1 genome is organised in two unique segments of different sizes: a long region (U_L, green) and a unique short region (U_S, blue). Each segment is flanked by a terminal and an internal sequence repeat (TR and IR respectively). The regions designated a' (dark blue) contains sequences than are necessary for packaging of progeny virions. (Adapted from Roizman 1996).

The IE gene products are the first viral proteins to be produced following the arrival of the viral DNA in the host cell nucleus. These IE proteins are necessary for initiating the expression of the E proteins from the β genes. The products of the β genes are responsible for viral DNA synthesis and further regulate the expression of γ proteins (γ1 "leaky" and γ2 true late genes). Both α and β gene products are required for the final expression of the γ genes which are structural proteins used in the virion progeny construction. Important late proteins include: VP16, the virion host shut-off protein (Vhs) responsible for the non-specific degradation of host m-RNA and blocking of host protein synthesis and ICP34.5, the viral neurovirulence factor responsible for establishment of infection in sensory neurons (Roizman, 1996).

1.9.6 Lytic life cycle of HSV1

As discussed in previous sections the nucleocapsid, surrounded by its tegument, arrives at the neuronal nucleus via retrograde transport. Once at the nucleus, the parental capsid attaches to a Nuclear Pore Complex (NPC) (Tognon *et al.*, 1981; McClain and Fuller, 1994; Spear, 2004). Electron microscope studies have shown that the viral DNA is quickly ejected from the NPC-bound parental capsid, through the nuclear pore and into the nucleoplasm, leaving behind the empty parental capsid that is eventually released in the cytoplasm (Tognon *et al.*, 1981; Ojala *et al.*, 2000). Once inside the nucleus, the viral DNA localises to sub-nuclear structures termed Nuclear Domain 10 (ND10, otherwise known as k-bodies or Pro-myelocytic leukaemia protein) (Burkham *et al.*, 1998). ND10 are discrete structures that are involved in a variety of cellular functions such as transcription, apoptosis or DNA repair (Borden, 2002; Bernardi and Pandolfi, 2003; Xu *et al.*, 2003) and are disrupted by a variety of other DNA viruses including adenovirus and papovavirus (Maul, 1998; Everett, 2001). In the case of HSV1, ND10 disruption occurs within 2 hours following infection (Maul *et al.*, 1993). The practical implication of the virally mediated disruption of the ND10 structures is complex. On the one hand, it is thought that this may serve to prevent the activation of antiviral genes by circulating interferon which is produced in response to the viral infection (Chee *et al.*, 2003). Alternatively,

ND10 may act either as nuclear depots for proteins kept in an inactive state until needed or as nuclear dumps for excess proteins awaiting degradation (Maul, 1998). The fact that HSV1 gene transcription occurs exclusively at ND10 following infection suggests that ND10 may provide the factors necessary for viral gene transcription to occur (Ishov *et al.*, 1997). ND10 have been likened to repositories for transactivating factors (Negorev and Maul, 2001). Thus, by disrupting the ND10 structures, HSV1 is in effect hijacking the machinery that is already in place in the host cell nucleus in order to transcribe its own genes (Maul, 1998; Roizman *et al.*, 2005). This would fit in with the ability of some viral proteins, such as the infected cell protein 0 (ICP0), to facilitate the expression of the remaining immediate early genes. Indeed, genetic analysis has revealed that ICP0 can directly modify the structure of the proteins comprising the ND10 structure and cause it to disperse (Maul *et al.*, 1993; Hagglund and Roizman, 2004).

The cascade of events leading to viral gene expression during the establishment of the lytic life cycle, are summarised in Figure 1.9.6-a (page 70). Overall, the cascade of expression starts with the production of IE proteins whose transcription is initiated by VP16 acting as a transactivator of IE gene promoters. VP16 cannot carry out this function alone as at it cannot directly bind to the viral DNA (Hughes *et al.*, 1999). It is therefore necessary for it to interact with host cell factors such as Oct1, a POU domain containing protein, and host cell factor (HCF) (Figure 1.9.6-a, panel **C**). The role of HCF is to import VP16 into the host cell nucleus (La Boissiere *et al.*, 1999) while Oct1 confers the ability to interact with the viral DNA. Via Oct1, the VP16/Oct1/HCF complex binds to a specific sequence on the viral DNA. This sequence has the motif TAATGA**R**AT (where R is a purine) and it is present just upstream of the TATA box element of all the IE genes promoters (Gaffney *et al.*, 1985). Oct1 interacts with the TAAT part of the motif (Gerster and Roeder, 1988) while the VP16 itself binds on the GARAT part (O'Hare and Goding, 1988). After binding of the VP16/Oct1/HCF complex, the C'-terminal transactivating domain of VP16 (Sadowski *et al.*, 1988) interacts with the host cell transcription factor IID (TFIID) and initiates the transcription of the IE genes (Klemm *et al.*, 1995).

The initiation of IE gene transcription heralds the beginning of the lytic life cycle of the virus. There are five IE proteins: ICP0, ICP4, ICP22, ICP27 and ICP47. ICP0 and ICP4 are responsible for the transcription of the β group of genes while ICP22 and ICP27 are required for the optimum expression of the γ genes (Roizman and Sears, 1996). The salient functions of the five IE genes are summarised below.

Gene	Protein	Alternative Name (kDa)	Function
α0	ICP0	Vmw110	Promiscuous transactivator. Optimal activity requires the presence of ICP4
α4	ICP4	Vmw175	Positively regulates the β and γ gene expression. Negatively regulates its own expression and the expression of ICP0. Also blocks apoptosis in infected cells
α22	ICP22	Vmw68	Regulatory protein, required for optimal expression of ICP0 and γ proteins
α27	ICP27	Vmw63	Negative regulator of α and positive regulator of γ genes expression
α47	ICP47	Vmw12	Stops antigen presentation to $CD8^+$ T cells

Vmw110, the gene encoding ICP0, maps to the short region repeats and therefore exists in two copies. Each copy of the gene is present in an antisense orientation to the latency associated transcripts (LAT) (Pereira *et al.*, 1977; Zhu *et al.*, 1991) (Figure 1.9.5-a, page 65) which is important in the role of ICP0 in the establishment and reactivation from latency (Wilcox *et al.*, 1997). ICP0 is not essential for the growth of the virus *in vivo* but disabling it negatively impacts on the efficiency with which it can propagate (Sacks and Schaffer, 1987; Everett, 1989; Chen and Silverstein, 1992; Cai and Schaffer, 1992). In cell lines infected with ICP0 deletion mutants (ΔICP0), especially at low multiplicities of infection (MOI), viral yields are 10 to 100-fold lower that those cell lines infected with wild type virus (Sacks and Schaffer, 1987; Everett, 1989). Transient transfection studies in which an ICP0 plasmid was co-transfected with different exogenous promoters, demonstrated that this protein is actually a promiscuous transactivator, capable of activating the transcription of non-viral genes (Everett and Dunlop, 1984; Everett, 1987). This transactivator property of ICP0 is enhanced in the presence of another IE protein: ICP4 (Everett, 1984; Gelman and Silverstein, 1985). Newly synthesized ICP0 accumulates at ND10

structures and as the levels of the protein increase they eventually occupy the whole of the nucleus. With further increases, ICP0 is displaced into the cytoplasm (Kawaguchi *et al.*, 1997; Lopez *et al.*, 2001) via a mechanism involving cyclin-D (Van Sant *et al.*, 2001). Since ICP0 cannot directly bind onto the viral DNA (Everett *et al.*, 1991), it is necessary for it to associate with selected cellular factors to carry out its effects. One of the proposed models for the way ICP0 promotes the expression of IE genes, attempts to identify this mechanism. It recently emerged that ICP0 contains an amino acid sequence that resembles the amino acid sequence present in the CoREST cellular protein (Gu *et al.*, 2005). CoREST is a cellular protein that by binding to another cellular protein termed REST, it can repress the expression of neuronal genes in non-neuronal cells (Andres *et al.*, 1999). The CoREST/REST complex exists in tight complexes with another protein HDAC, in both neuronal and non-neuronal cells. It is thought that this complex can lead to the complete silencing of target genes (Lunyak *et al.*, 2002; Ballas *et al.*, 2005). Importantly, CoREST/REST and HDACs are found in complexes in uninfected neurons. However, when those neurons become infected with wild type HSV1, which of course expresses high levels of ICP0, a different picture emerges. In these cells, 50% of the CoREST/REST/HDAC complexes are dissociated (Gu *et al.*, 2005). It is possible that this effect of ICP0 could lead to the reversal in the repression of viral gene expression within the host cell (Figure 1.9.6-a, panel **C**, page 70). This hypothesis provides a plausible explanation for the mechanism by which ICP0 initiates the expression of the α genes (Roizman *et al.*, 2005). The ICP4 gene (Vmw175) is located in the terminal repeats of the U_S region of the viral genome. ICP4 is another essential protein of HSV1 and an important transcriptional regulator (DeLuca *et al.*, 1985). ICP4 binds as a homodimer (Metzler and Wilcox, 1985) onto viral DNA sequences with the conserved motif: **R**TCGTCNN**Y**N**Y**S**G**, where R is a purine, Y is a pyrimidine while S represents either a C or G nucleotide (N is any nucleotide) (DiDonato *et al.*, 1991). The C′-terminal region of ICP4 then recruits the TATA binding transcription activation factor TFIID (Carrozza and DeLuca, 1996). The binding of TFIID facilitates the subsequent recruitment of TFIIB and polymerase-I and transcription is then initiated (Grondin and DeLuca, 2000).

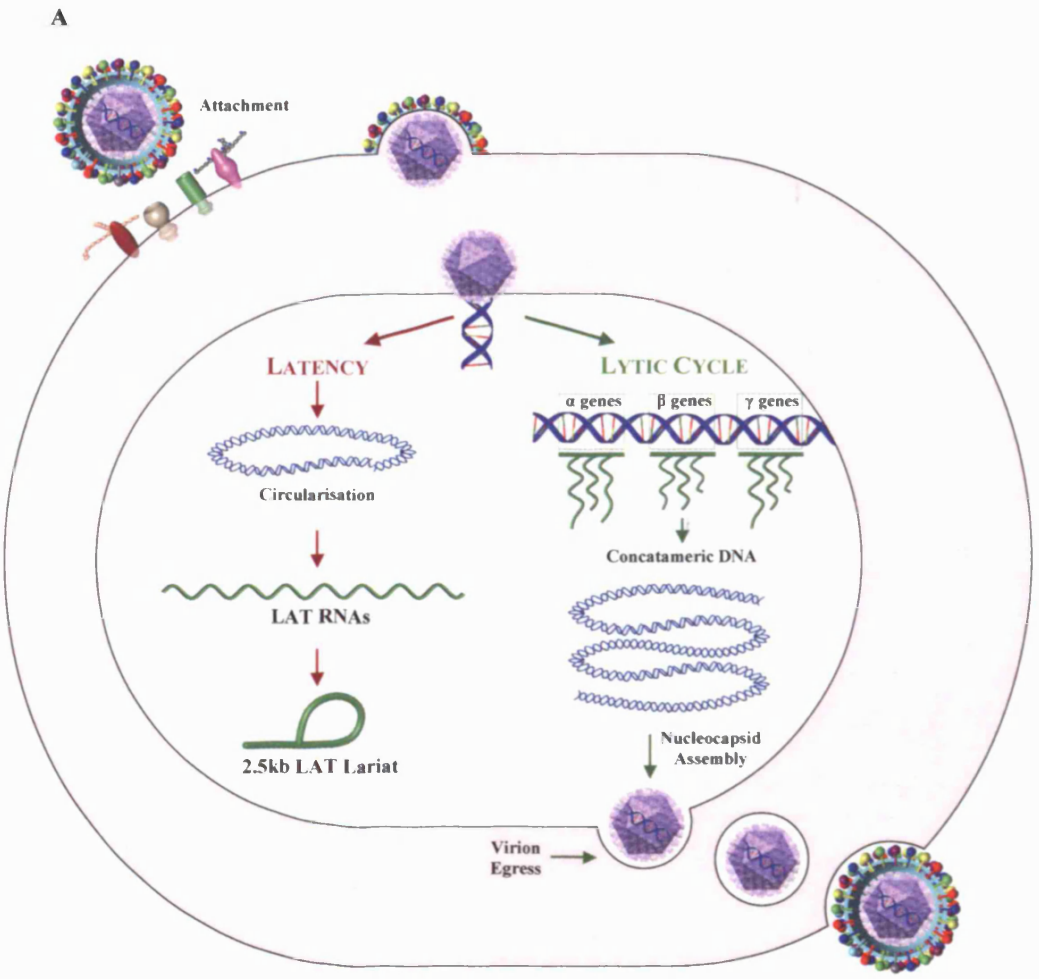


Figure 1.9.6-a: Viral gene expression during the HSV1 lytic cycle & latency.

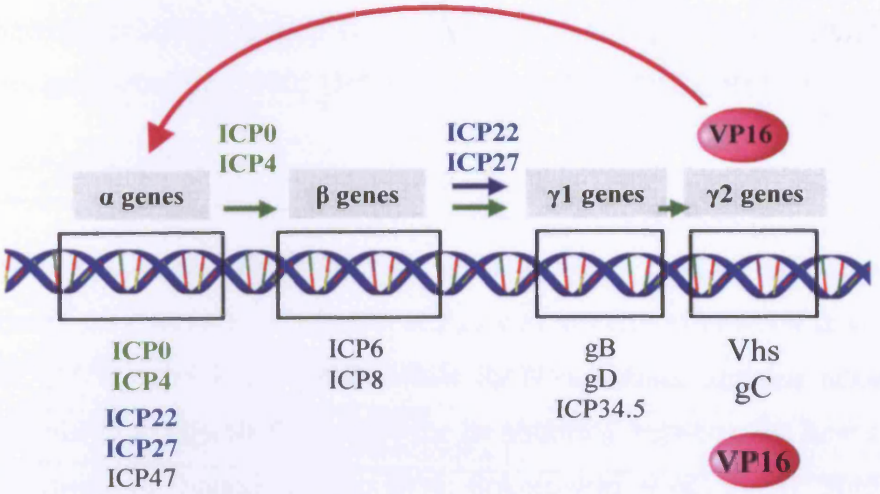
A. Stages of the HSV1 life cycle. Following the arrival of vDNA into the cell nucleus the virus will either enter the quiescent state of latency or initiate a lytic life cycle. During latency, the dsVDNA circularizes and there is no expression of any viral proteins. Only the LAT mRNA transcripts are produced. During the lytic cycle, IE, E and L gene expression ensues. Concatameric, dsVDNA is cleaved and packaged into new virions (Glorioso *et al.*, 1995).

B. IE gene expression occurs in a well-controlled cascade manner. The tegument component VP16 transactivates the expression of the IE proteins ICP0 and ICP4. ICP0 and ICP4 are necessary for the expression of the E and L genes as they act as transactivators for their promoters. The ICP22 and ICP27 IE genes are required for the efficient production of the L genes (Roizman, 1996; Roizman *et al.*, 2005).

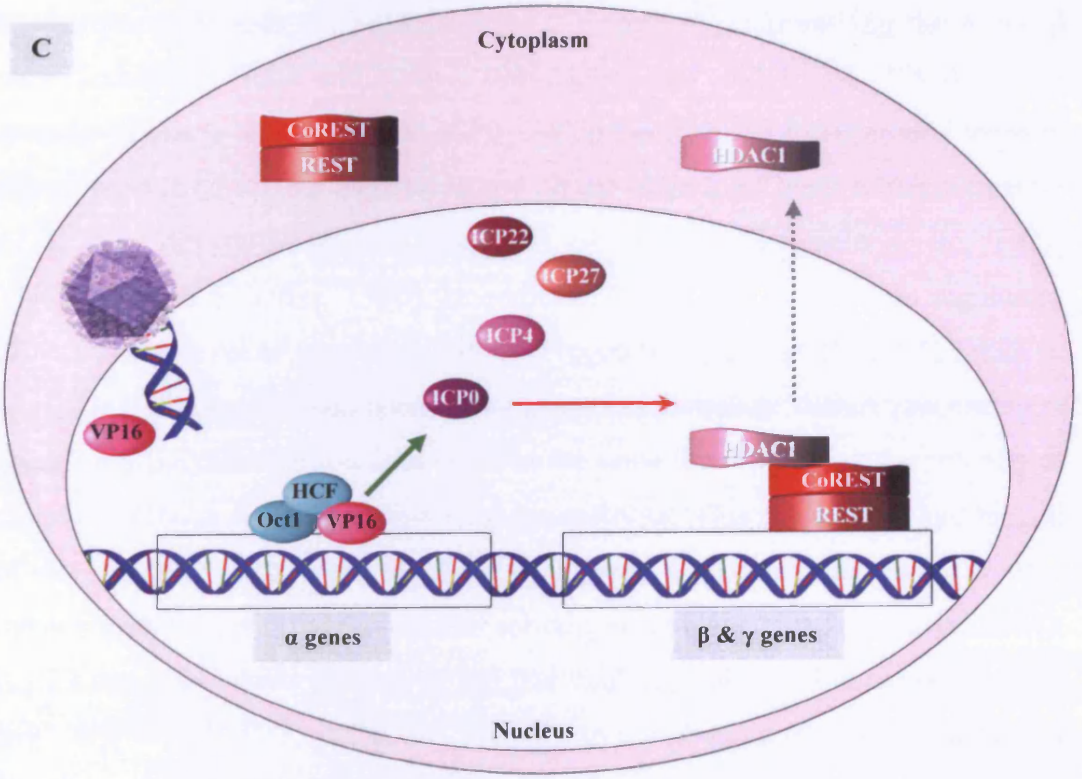
C. Proposed model for the control of IE gene expression. Upon entry of the viral DNA into the host cell nucleus, the tegument component VP16 associates with cellular factors such as Oct1 and HCF and initiates the transcription (A, green arrow) of the IE genes: ICP0, ICP4, ICP22, ICP27 and ICP47 (Roizman and Sears, 1996). ICP0 causes the dissociation of HDAC1 from the CoREST-REST complex (red arrow) while the subsequently produced E and L viral proteins cause the translocation of the HDAC1 and CoREST into the cytoplasm (dotted arrow), thus alleviating the transcriptional repression these molecules exert on the viral genome (Roizman *et al.*, 2005).

Diagrams: **A** adapted from Glorioso *et al.* (1995), **B** & **C** adapted from Roizman *et al.* (2005).

B


ICP0
ICP4
ICP22
ICP27
VP16
α genes
β genes
γ1 genes
γ2 genes
ICP0
ICP4
ICP22
ICP27
ICP47
ICP6
ICP8
gB
gD
ICP34.5
Vhs
gC
VP16

C


Cytoplasm
CoREST
REST
HDAC1
ICP22
ICP27
ICP4
ICP0
VP16
HCF
Oct1
VP16
HDAC1
CoREST
REST
α genes
β & γ genes
Nucleus

Being a transcriptional activator ICP4 is responsible for the transactivation of the remaining E and L genes during the lytic cycle. In addition, ICP4 represses the activity of latency promoter and down-regulates its own expression and that of ICP0 (Dixon and Schaffer, 1980; DeLuca and Schaffer, 1985; Roberts *et al.*, 1988).

ICP27 (Vmw63) is another essential viral protein with a number of different regulatory functions (Sacks *et al.*, 1985). ICP27 can directly bind RNA through its **R**GG-box (Mears and Rice, 1996), while its N'-terminus contains nuclear export and localisation signals that allow for its shuttling between the host cell nucleus and cytoplasm (Sandri-Goldin, 1998; Sokolowski *et al.*, 2003). Studies utilising ICP27 mutants have demonstrated that ICP27 is necessary for efficient DNA replication (Sekulovich *et al.*, 1988; Estridge *et al.*, 1989; McCarthy *et al.*, 1989; Larralde *et al.*, 2006). In the initial stages of infection ICP27 favours the expression of E proteins but in the later stages it switches the emphasis of transcription towards the production of L proteins by suppressing the α and β gene promoters (Rice and Knipe, 1990; Uprichard and Knipe, 1996). ICP27 directly interacts with ICP4 and ICP0. This interaction on the one hand leads to the repression of E gene expression and on the other hand leads to the activation of L gene transcription (Sekulovich *et al.*, 1988; Hardwicke *et al.*, 1989; McMahan and Schaffer, 1990). In addition, ICP27 can exert some regulatory effects at the level of post-transcriptional regulation (Jean *et al.*, 2001; Smith *et al.*, 2005). The protein has been demonstrated to stimulate 3'RNA processing at some viral polyadenylation sites while at the same time inhibiting the splicing of cellular mRNAs and susceptible viral pre-mRNAs. This favours the production of viral proteins as the majority of the viral genes, unlike the host, do not contain introns and do not interact with the splicing machinery (Sandri-Goldin, 1994). ICP27 can also inhibit splicing of the few viral pre-mRNAs that contain introns (e.g.: ICP34.5, ICP22, and ICP0) but only in the absence of other viral factors (Lindberg and Kreivi, 2002). The mechanism of this is still unclear but ICP27 appears to be capable of differentiating intron-containing from intronless viral pre-RNAs. ICP27 facilitates the quick nuclear export of intronless viral mRNAs (Smith *et al.*, 2005).

ICP22 (Vmw68) is a non-essential viral protein and has been shown to be dispensable for viral growth in some cell lines (Poffenberger *et al.*, 1993; Rice *et al.*, 1995). The function of this protein is not totally understood (Roizman and Sears, 1996) but its presence has been shown to boost the efficiency of L gene expression (Poffenberger *et al.*, 1994; Orlando *et al.*, 2006). It has been suggested that ICP22 has two separate functions, one linked to a unique N'-terminal amino acid sequence (Ogle and Roizman, 1999; Cun *et al.*, 2006). On the one hand, it has been reported that ICP22 is involved in the production of an aberrantly phosphorylated form of RNA polymerase II that is capable of both repressing host cell transcription (Rice *et al.*, 1995; Spencer *et al.*, 1997; Long *et al.*, 1999) and increasing the stability of ICP0 mRNA (Carter and Roizman, 1996). In addition, it has been suggested that ICP22 may be directly involved in viral replication as it has been repeatedly shown to localise in ND10 structures (Leopardi *et al.*, 1997; Stelz *et al.*, 2002).

The final IE gene, ICP47 (Vmw12) is not essential for viral growth in cell lines and does not have a regulatory function (Mavromara-Nazos *et al.*, 1986). Its main function is to inhibit antigen presentation to $CD8^+$ T lymphocytes, therefore enabling the virus to escape immune surveillance mechanisms (Goldsmith *et al.*, 1998). ICP47 achieves this by binding to the transporter of antigen processing (TAP) (Hill *et al.*, 1995) complex thus preventing it being loaded with viral proteins onto MHC Class I molecules (Bauer and Tampe, 2002). The role of ICP47 in immune evasion, as well as the involvement of the immune system in the longevity of the virally mediated foreign gene expression, is further examined in Chapter 7. Following the expression of IE genes the lytic cycle progresses with the E and L genes. The E genes are responsible for viral DNA replication and encode proteins such as ICP8, a major DNA binding protein, thymidine kinase (TK), a ribonucleotide reductase (RR) and various viral polymerases (Roizman and Sears, 1996; Devi-Rao *et al.*, 1997; Wagner and Bloom, 1997). Viral replication occurs in the ND10 structures (Ishov and Maul, 1996) and, despite earlier reports, it has recently been demonstrated that the viral DNA does not circularise in order for replication to occur in the lytic cycle (Jackson and DeLuca, 2003).

The last genes to be transcribed are the L genes (Figure 1.9.6-a, panel **A,** page 70). These encode structural components of the capsid, tegument and envelope including Vhs, VP16, envelope glycoproteins and ICP34.5, the neurovirulence factor. The transactivators ICP0 and ICP4 activate the L promoters. However, completion of viral DNA replication is a prerequisite for the initiation of L gene transcription (Roizman and Sears, 1996). Unlike earlier reports, it is now clear that the viral DNA does not circularise during the lytic cycle (Jackson and DeLuca, 2003). Following replication, the newly synthesised viral DNA is cleaved into functional units based on the presence of the a' sequences located in the repeat regions of the genome (Figure 1.9.5-a, page 65) and is subsequently packaged into new capsids. Once assembly is completed the nucleocapsids acquire their tegument proteins including vhs and VP16. The tegument proteins aid the exit of the nucleocapsid from the nucleus and its subsequent envelopment (Smibert *et al.*, 1994; Mossman *et al.*, 2000). As tegument surrounded nucleocapsids bud through the inner nuclear membrane and into the cytoplasm, they acquire their glycoprotein-studded envelope.

The route that progeny virions follow in order to exit the cell has been the subject of considerable research and two alternative pathways have been proposed. Firstly, the re-envelopment pathway (Granzow *et al.*, 2001) suggests that the enveloped virions exiting the inner nuclear membrane loose their envelope as they fuse with the outer nuclear membrane and therefore non-enveloped virions are released into the cytoplasm. The naked capsids pass through the Golgi apparatus, are packaged into secretory vesicles and are finally released into the extracellular space. Secondly, the luminal pathway (Enquist *et al.*, 1998) suggests that virions exit the nucleus with their envelope and pass through the ER and cytoplasm where the envelope glycoproteins are further processed and adopt their mature form. Mature enveloped virions are finally released in the extracellular space via the secretory route (Roizman and Sears, 1996).

1.9.7 The Latent life cycle

Normally, once the viral DNA arrives at the nucleus of sensory neurons, it does not follow the lytic life cycle. Instead, it enters into a latent life cycle. During latency, the viral DNA circularises and becomes an episomal, quiescent structure (Honess *et al.*, 1989). During latency, transcription of viral genes is silenced and no viral gene proteins or new virions are produced. Unlike the beta and gamma herpes viruses, HSV1 viral DNA does not use methylation as a means of silencing viral gene expression (Dressler *et al.*, 1987; Kubat *et al.*, 2004). There is however a small region of the viral DNA that remains transcriptionally active. This region, termed the latency associated transcript (LAT) region is located in the repeats of the U_L segment of the viral genome. The LAT region produces a group of RNA transcripts named latency associated transcripts (LATs). Their presence has been confirmed by *in situ* hybridisation studies in animal models and human trigeminal ganglia (Deatly *et al.*, 1987; Croen *et al.*, 1987). Interestingly, LATs are transcribed in an antisense orientation to that of ICP0 (Stevens *et al.*, 1987; Croen *et al.*, 1987). This led to the suggestion that the production of LATs inhibits the expression of ICP0, which as discussed favours viral gene expression and the lytic life cycle (Stevens *et al.*, 1987; Farrell *et al.*, 1991; Ahmed and Fraser, 2001).

LATs exist as a mixed population of a large 8.3kb long and the highly abundant 2 kb and 1.5kb stable introns, resulting from the splicing of the larger LAT (Figure 1.9.5-a, page 65) (Wagner *et al.*, 1988; Dobson *et al.*, 1989; Zwaagstra *et al.*, 1990; Mitchell *et al.*, 1990). The largest 8.3kb LAT is termed primary LAT and appears to be polyadenylated and is highly unstable. However the most abundant LAT species are the smaller 2 kb and 1.5kb LATs, which remain non-polyadenylated and uncapped within neuronal cells (Stevens *et al.*, 1987; Wagner *et al.*, 1988; Devi-Rao *et al.*, 1991). Within the vicinity of the 2 kb LAT there are approximately three ORFs that encode a 273 aa sequence which is highly conserved between different HSV1 strains (Coffin *et al.*, 1998). The fact that this 2 kb LAT has been found to associate with splicing factors in the host cell may suggest that either the 2 kb LAT may have some regulatory function or that ORF encodes a protein that may have some regulatory properties (Nicosia *et*

al., 1994; Goldenberg *et al.*, 1997; Ahmed and Fraser, 2001). This however still remains only a hypothesis as deletion of the 2 kb LAT ORF has no effect on virus physiology (Fareed and Spivack, 1994). A study conducted in our laboratory demonstrated that artificially over-expressing the 2 kb LAT-ORF, results in improved viral growth, even in cell lines that are normally non-permissive. An increased ability to overcome any deficiencies in the ICP0 expression was also demonstrated (Thomas *et al.*, 1999). Interestingly, when frame shift mutations were introduced in the LAT-ORF and over-expression experiments were repeated, there was no evidence of improved growth while the ability to compensate for ICP0 deficiencies was lost. Finally, when *in vitro* transcription/translation reactions were performed, a 30 kDa protein was detected that was absent when frame shift mutations were introduced in the LAT-ORF. Thomas *et al.* (1999) went on to propose that the localisation of the LAT ORF-encoding RNA is modified during reactivation, so that its translation is possible. This LAT-ORF protein could then compensate for the antisense effect exerted on ICP0 during latency and thus contribute to the reactivation process (Thomas *et al.*, 1999). During latency, there are two promoters that remain active: latency active promoter-1 (LAP1) and latency active promoter-2 (LAP2). In this part of the introduction, the features of these two promoters are examined. The various attempts at exploiting those features in order to drive the expression of exogenous genes will be further explored in Chapter 3.

Each of the LAP promoters is capable of functioning independently from one another (Dobson *et al.*, 1989; Goins *et al.*, 1994; Jones, 2003). LAP1 is located approximately 700bp upstream from the 5' end of the 2 kb LAT. Transcription is initiated 28bp downstream of the TATA element and it has been demonstrated that it is the principal latency promoter (Dobson *et al.*, 1989; Zwaagstra *et al.*, 1990). LAP1 is active in both neuronal and non-neuronal cells but its activity is somewhat lower in the non-neuronal cell lines, a feature that has been attributed to the inhibition of sequences upstream of the TATA element (Batchelor and O'Hare, 1990; Zwaagstra *et al.*, 1990). The LAP1 is thought to contain a variety of control elements including a sequence element that resembles an ICP4 binding site, located upstream of the primary LAT cap site. These control elements in the

LAP1 could interact with both negative and positive regulatory signals. Nicosia *et al.* (1993) were the first to propose the existence of a second LAT promoter since they reported that the 2 kb LAT could still be produced even when the TATA element of LAP1 was absent (Nicosia *et al.*, 1993). LAP2 is mapped downstream of LAP1 and 58bp upstream of the 5' end of the 2 kb LAT (Goins *et al.*, 1994). LAP2 is a TATA-less promoter and although it is classified as a latency-associated promoter, it is primarily active during productive infections (Chen *et al.*, 1995). In addition, it contains neuronally active elements such as E2F sites (Goins *et al.*, 1994) which have been suggested to increase the activity of this promoter. Interestingly, LAP2 shares a sequence homology with eukaryotic housekeeping and oncogene promoters, which are also TATA-less and contain elements such as CT rich motifs or poly-T elements that can potentially alter chromatin structure (Reynolds *et al.*, 1984; Valerio *et al.*, 1985). It has been proposed that alteration of the chromatin structure is the mechanism by which LAP2 influences the activity of LAP1. Deletion mutants where LAP1 or LAP2 were abolished during latency, showed that there was a 500 fold decrease in the levels of the 2 kb LAT when the LAP1 was not functioning as opposed to a 3 fold decrease when the deletion affected LAP2 alone. It therefore follows that even though LAP1 is the primary latency promoter, responsible for enhanced activity in neurons, the weaker LAP2 promoter is also required for the optimum production of LATs (Goins *et al.*, 1994; Chen *et al.*, 1995). In addition it was shown that LAP1 can not support the long term expression during latency in DRG neurons without the presence of LAP2 (Lokensgard *et al.*, 1997). This was taken to suggest that there was a long-term expression element in the LAP2 region. Lokensgard and colleagues (1997) proposed that this element overlaps LAP2 and part of the 2kb LAT intron. Furthermore, it was reported that this element could act in a bi-directional manner to confer long-term activity on LAP1 from the gC locus of the viral genome (Berthomme *et al.*, 2000). In our laboratory we have shown that it is possible to exploit these sequences present in LAP2 to confer long term activity on non-LAT promoters such as the CMV promoter (Palmer *et al.*, 2000; Lilley *et al.*, 2001b).

1.9.8 HSV1 and gene therapy for the nervous system

Wild type virus is highly pathogenic and cannot therefore be administered to the CNS by direct inoculation. Hence, in order for a potentially useful gene delivery vector system to be of use, this potentially lethal cytotoxicity should be eliminated. In addition, the particular requirements for any potentially therapeutic gene to have any meaningful benefit in the injured CNS it is necessary to utilise suitable promoters that will be able to sustain its expression in the long term (Lilley *et al.*, 2001a).

There are two types of HSV vectors, which have been used as gene therapy vectors. The first approach is based on the construction of disabled HSV1 vectors where the transgene is recombined into the viral genome, which has previously been deleted for any potentially cytotoxic viral genes. The second approach relies on the use of defective vectors termed amplicons (Frenkel, 2006; Tyler *et al.*, 2006). Amplicon vectors are created from plasmids that contain the transgene, an HSV1 packaging signal and an HSV1 origin of replication (Spaete and Frenkel, 1982). The plasmid is co-transfected with a defective HSV1 helper virus into a cell line that complements the disability of the helper virus. The plasmid then replicates in fragments of up to a full viral genome length relying on the machinery from the helper virus acting on the HSV1 origin of replication in the amplicon plasmid. These newly formed concatamers are packaged into the progeny capsids but this can only be successful in the presence of the helper virus. The progeny harvested will be a mixture of virion containing the original helper virus genome and amplicon virions, which will have packaged concatameric plasmid sequences. A variety of defective helper viruses have been used in this system. These included virus constructs with temperature sensitive mutations or deletions in essential genes such as ICP4 (Geller and Breakefield, 1988). Although amplicons could be used to deliver genes to neuronal cells, their wide spread use has been hindered by the fact that high titre stock production is hard to achieve and stocks were often contaminated with the cytotoxic helper virus (Kwong and Frenkel, 1985; Lowenstein *et al.*, 1994). The few applications of these vectors in the nervous system that have been reported are focused on transducing cerebral neurons (Tyler *et al.*, 2006).

This thesis and the attempts at inducing regeneration in the injured CNS milieu are based on the use of highly disabled HSV1 vectors. The vectors utilised here lack a combination of essential and non-essential genes, which significantly impairs the innate cytotoxicity of the virus. In addition, this vector system makes use of especially designed cassettes that fully exploit the ability of HSV1 for low level transcription during latency (Lilley *et al.*, 2001a).

CHAPTER 2.0

MATERIALS & METHODS

2.1 Materials

2.1.1 Suppliers

Amersham International Plc. (Amersham Place, Little Chalfont, Buckinghamshire, HP7 9NA, UK). Rainbow™ coloured protein molecular weight markers, α-^{32}P-dCTP (3000Ci/mM), Hybond™-C^{+} & Hybond™-N nitrocellulose membranes, N,N'-methylene-bis-acrylamide, ECL™ western blotting detection system.

BD Biosciences. (The Danby Building, Edmund Halley Rd, Oxford science Park, OX4 4DQ, UK). Yeast extract, Bacto®-agar, Bacto®-tryptone.

Berkley Antibody Company (BAbCo) Ltd. (1223 south 47th Street, Richmond, CA, 94804, USA). Anti-TUJ1 specific antibody (section 2.1.6).

Cayla (Toulouse, France) Zeocin™, Phleomycin derivative.

COHESION Technologies Inc. (2500 Faber Place, Palo Alto, California 94303, USA). Vitrogen™ Collagen.

Dako Ltd. (Cambridge House, St Thomas Place, CB7 4EX, UK). Rhodamine and HRP conjugated antibodies.

Gelman/Pall Life Sciences Inc. (Wagner Road, Ann Arbor, MI, Washtenaw County, USA). 0.45μm and 0.20μm disposable filters.

Gibco-BRL Life Technologies Ltd. (Invitrogen: 3 Fountain Drive, Inchinnan Business Park, Paisley, PA4 9RF, UK). Tissue culture media, solutions and supplements for fibroblast and primary neuronal cultures, G418 (Neomycin), 1Kb DNA ladder.

Greiner Ltd. (Brackmills Business Park, Caswell Rd, Northampton, NN4 7EZ, UK). General disposable plasticware.

Insight Biotechnology Ltd. (P.O. BOX 520, Wembley, Middlesex, HA9 7YN, UK). 4-chloro, 5-bromo, 3-indolyl-β-galactosidase (X-Gal).

Invitrogen - Life Technologies UK. (3 Fountain Drive, Inchinnan Business Park, Paisley, PA4 9RF, UK). Murine Nerve Growth Factor (NGF), 2.5S Subunit.

MERCK Chemicals Ltd. (Padge Road, Nottingham, NG9 2JR, UK). Analytical grade laboratory chemicals.

NEN - Life Science Products (549 Albany Str., Boston, Massachusetts, 02118-2512, USA). NEN™ Kit - Tyramide Signal Amplification

Nalge Nunc Inc. (75 Panorama, Creek Drive, Rochester, NY, 14625-2385, USA). Plastic-ware for tissue culture purposes.

Pfizer Inc. (Walton Oaks, Dorking Rd., Tadworth, Surrey, KT20 7NS, UK). Ready-beads for radioactivity labelling of plasmid DNA probes.

Promega Inc. (Delta House, Southampton Science Park, Southampton, SO16 7NS, UK). Restriction and DNA modifying enzymes and respective buffer solutions. Recombinant CNTF.

Qiagen Ltd. (Fleming Way, Crawley, West Sussex, RH10 9NQ, UK). Qiagen™ Midi-prep plasmid DNA kits.

Quadratech Ltd. (P.O. BOX 167, Epsom, Surrey, KT18 7YL, UK). Cholera – toxin Subunit B – HRP Conjugate.

R&D Systems Eyrope Ltd. (19 Barton Lane, Abington Science Park, OX14 3NB, UK). Recombinant human NT3.

Santa - Cruz Biotechnology Inc. (Insight Biotechnology Ltd, P.O. BOX 520, Wembley, HA9 7YN, UK). Antibodies to rat NT3, rat CNTF, mouse IL10 and rat BDNF.

Sera – Tech Biologicals Inc. (900 Bugg Ln Suite 101, San Marcos, TX 78666)
Normal goat serum and normal horse serum, Streptavidin-HRP antibodies.

Serotec Ltd. (Oxford Science Park, UK). Anti-rat OX42.

Sigma-Aldrich Company Ltd. (The Old Brickyard, New Road, Gillingham, Dorset, SP8 4XT). Analytical grade laboratory chemicals, recombinant rat Cilliary Neurotrophic Factor (CNTF), BS3 Bis(sulfosuccinimidyl)suberate protein cross - linking agent.

Sterilin Ltd. (Pengam Road, Aberbargoed, Pontypridd, CF81 9FW, UK). General disposable plasticware.

Stratagene Europe Ltd. (Gebouw Texas, Hogehilweg 19, 1101 CB Amsterdam Zuidoost, Netherlands). *E.Coli* XL1-Blue bacterial strain.

VECTOR Laboratories Ltd. (3 Accent Park, Bakewell Road, Orton Southgate, Peterborough, PE2 6XS, UK). Biotinylated antibodies and Vectastain™ ELITE ABC (avidin – biotin) Kit

Whatman International Ltd. (Whatman House, St. Leonard's Road, Maidstone, Kent, ME16 0LS, UK). 3MM chromatography paper, Plydisc™ HD disposable 5µm filters.

2.1.2 Animals

All the animals used in this study (rats: LEWIS and Fischer 344, mice: Balb/c and SCID) were obtained either from breeding colonies within the Department of Biological services, UCL or from Harlan Laboratories, Oxon, UK. Animal Surgery was carried out according to approved protocols (Home Office project licence No: PPL70/4293).

2.1.3 Primers

Oligonucleotide primers were constructed by Genosys (Pampsiford, UK).

Primer	Sequence (5'→3')
Rat NT3 Forward	GGTGCTGTCACTCAGCAGGACCCGGGG
Rat NT3 Reverse	CCCCGGGTCCTGCTGAGTGACAGCACC

2.1.4 Bacterial strains

The bacterial strain used for the molecular biology aspect in this thesis was *E.Coli* XL1-Blue, Genotype: *RecA1 endA1 gyrA96 thi-1 hsdR17 supE44 relA1 lac* [*F'proAB Lacl*^{q} *ZΔM15, Tn10 (Tet)*^{r}] (Stratagene Ltd).

2.1.5 Cell lines

ATCC®* is the trademark of the American Type Culture Collection

Cell Line	Origin	Culture Media	Source
BHK 21 Clone 13	Baby Hamster Kidney cells	1x DMEM 100U/ml Penicillin 100µg/ml Streptomycin 10%(v/v) FCS 5%(v/v) TPB	Macpherson *et al.* 1962 ATCC®* CCL 10
27/12/M:4	BHK-based complementing vm65, ICP4 and ICP27 deletions	As for BHK but further supplemented with: 800µg/ml G418 750µg/ml Zeocin	Thomas et al. 1999

1°Antibody	Dilution	2°Antibody	Source
Mouse TUJ1 anti-neuronal specific tubulin (Cat. No: MMS-435P, Stock: 1 mg/ml)	1:5000	Rhodamine conjugated anti–mouse Ig, 1:100	BabCo, USA
Mouse anti-rat OX-42, clone CD11 IgG (Cat. No: MCA275B, Stock: 0.1 mg/ml)	1:200	Biotinylated horse anti mouse IgG, rat absorbed	Serotec, UK
Monoclonal anti-GFAP clone GA5 (Cat. No: G3893, ascitic fluid)	1:600	Biotinylated horse anti mouse IgG, rat absorbed	Sigma - Aldrich Company, UK
Rabbit polyclonal anti-rat NT3 (N-20) (Cat. No: SC-547, Stock: 200 µg/ml)	1:200	HRP - conjugated anti-rabbit 1:1000	Santa Cruz Biotechnology , USA
Goat polyclonal anti-mouse IL-10 (M18) (Cat. No: SC-1783, Stock: 200 µg/ml)	1:200	HRP – conjugated anti-goat 1:1000	
Polyclonal, rabbit anti-SCG10	1:3000	Biotinylated goat anti rabbit IgG, rat absorbed	Dr G. Grenningloh (University of Lausanne Switzerland)
Rabbit polyclonal anti-rat BDNF (N-20) (Cat. No: SC-546, Stock: 200 µg/ml)	1:200	HRP - conjugated anti-rabbit 1:1000	Santa Cruz Biotechnology, USA
Goat polyclonal anti-rat CNTF (R-20) (Cat. No: SC-1912, Stock: 200 µg/ml)	1:200	HRP – conjugated anti-goat 1:1000	

2.1.7 Standard buffers

TE Buffer:
10mM Tris-HCl pH 8.0
1mM EDTA pH 8.0

TAE Buffer:
400mM Tris Base
200mM Sodium Acetate
20mM EDTA pH 8.3

0.4M PBS Buffer:
10.44g $NaH_2PO_4(H_2O)$
46g Na_2HPO_4(anhydrous)
up to 1ltr and 36g Sodium Chloride

0.1M TBS Buffer:
13.22g Tris-HCl
1.94g Tris Base
9g Sodium Chloride up to 1ltr

SSC:
150mM NaCl
15mM Sodium Citrate

Luria Bertani (LB) media:
1% (w/v) Bacto®-tryptone
1% (w/v) NaCl
0.5% (w/v) Bacto®-yeast extract
LB media was autoclaved prior to use at 120°C for 20 min at 10lb/square inch (psi).

Chloroform/IAA:
96%(v/v) Chloroform
96%(v/v) Isoamyl Alcohol

TNT Buffer:
10mM Tris-HCl pH 7.5
15mM NaCl
0.05% Tween 20

SSC:
150mM NaCl
15mM Sodium Citrate

Equilibrated Phenol:

Liquefied phenol was equilibrated with an equal volume of 0.1M Tris-HCl pH8.0. An average of two extractions were required for a 500ml batch. Equilibrated phenol was stored at 4°C in a dark room and was discarded one month after equilibration.

2.2 Methods

2.2.1 Propagation of bacterial strains

Bacteria were grown either on solid agar plates containing 2% Bacto®-agar or liquid Luria – Broth (LB). Both were supplemented with 100 μg/ml Ampicillin when selection was required. Agar plates were incubated overnight at 37°C in a standard incubator. Liquid cultures were incubated overnight at 37°C in an orbital shaker at 200rpm.

2.2.2 Transformation of bacteria

A single colony of *E. Coli* XL1-Blue was inoculated in 15ml of LB containing no antibiotic and incubated at 37°C in an orbital shaker at 200rpm, overnight. 100μl of the overnight culture were used to inoculate 100ml of LB containing no antibiotic and returned to the orbital shaker until bacterial growth reached an OD_{580} of approximately 0.5 indicating that growth was in a log phase. A standard calcium chloride technique was used to prepare competent cells. Bacteria were then pelleted by centrifugation at 3000rpm for 10 minutes. The supernatant was then discarded and the bacteria were re-suspended in 10ml of ice cold 100mM $CaCl_2$, pelleted as before and finally re-suspended in 4ml of ice cold $CaCl_2$. Competent cells were stored on ice until required and used no more than 72 hours after preparation. Competent cells were transformed by addition of DNA and subsequent incubation on ice for 30 minutes. The cells were then heat-shocked for 90 seconds at 42°C and returned on ice for a further two minutes. 800μl of LB was added and after a brief, one-hour incubation at 37°C/200rpm, bacteria were pelleted, re-suspended in 100μl of LB and plated onto selective agar medium containing ampicillin at 100μg/ml. If detection of β-galactosidase activity was required, bacteria were plated onto agar plates containing 50μl of a 20mg/ml stock of X-GAL made up in dimethyl formamide(DMF). Plates were incubated at 37°C, overnight in a standard incubator.

2.2.3 Small scale plasmid DNA extraction

Single colonies of *E. Coli* XL1-Blue, transformed successfully with the plasmid of interest, were inoculated in 3ml of Luria Broth (LB) containing ampicillin at 100μg/ml and incubated overnight at 37°C in an orbital shaker at 200rpm. 1.5ml of the overnight culture was spun down for one minute and 100μl of solution I (50mM Tris-HCl pH7.5, 10mM EDTA, 100μg/ml RnaseA), 200μl of solution II (200mM NaOH, 1%($^{v}/_{v}$) Triton x100) and 150μl of solution III (3M KOAc pH4.8) were sequentially added and thoroughly mixed by vortexing. The resulting protein pellet was discarded and the DNA was precipitated with 500μl of isopropanol. It was then washed twice with 70% ethanol, dried and re-suspended in 50μl of dH_2O containing 20μg/ml RnaseA. The DNA was stored at -20°C.

2.2.4 Large scale plasmid DNA extraction

Midi-Preps were carried out using the QIAGEN©-Tip 100 columns, according to the manufacturer's protocol. A single bacterial colony, picked from an agar plate, was inoculated into 500ml of LB supplemented with the appropriate antibiotic and incubated overnight at 37°C in an orbital shaker at 150rpm. 100ml of the resulting culture were centrifuged at 3500 rpm for 10 minutes. The average yield from a 100ml overnight culture was 1 μg/μl of DNA (about 100 μg in total).

2.2.5 Agarose gel electrophoresis

Low melting point (LMP) Agarose gels 1%(w/v) and normal agarose 0.08%(w/v) gels containing 0.5μg/ml of Ethidium Bromide were run in 1x TAE buffer at 50 and 100 Volts respectively. Approximately 0.1 volume of 10x loading buffer was added to the samples prior to loading. The bands were visualised using a UV trans-illuminator and photographed using a Polaroid camera and film. A 1 kilobase (kb) ladder was used as a marker to identify band sizes on the gel.

2.2.6 Quantification of DNA

Quantification of plasmid DNA was performed following DNA extraction. 5µl of purified DNA were diluted 200 fold into a total volume of 1ml ddH_2O and the absorbance was read at A260 using a calibrated spectrophotometer. The DNA content was then calculated using the formula below, which is based on the fact that an A260 of 1 corresponds to 50µg/ml.

$$[DNA] = \frac{(A260 \times 200 \times 50)}{1000} \mu g\ ml^{-1}$$

2.2.7 DNA sequencing

Plasmid DNA was purified using phenol/chloroform. DNA at a concentration of 50ng/µl was sequenced by the Dept. of Biochemistry (sequencing facility), University of Cambridge, UK. The primers for each case were used at a concentration of 10pmol/µl.

2.2.8 Purification of plasmid DNA by phenol extraction

The total volume of the DNA digest was adjusted to 400µl with dH_2O. An equal volume of phenol:chloroform:isoamyl alcohol(IAA) (24:24:1) was added. The mixture was thoroughly vortexed and then centrifuged at 14,000 rpm for 2 minutes. The top layer consisting of the aqueous phase was removed and the above step was repeated until the interface appeared clear. Once this was achieved, the aqueous phase was mixed with an equal volume of chloroform:IAA and pulse spun. After spinning at 14,000 rpm for 2 minutes, the aqueous layer was again removed and mixed with 40µl of 3M Sodium Acetate (pH 5.5) and 880µl of ice cold 100% Ethanol. DNA was allowed to precipitate by an one-hour incubation at -70°C. The DNA was pelleted by a 15 min centrifugation step at 14,000 rpm. The supernatant was then discarded and the pellet was washed twice with 70%(v/v) Ethanol. The DNA was allowed to dry and it was finally re-suspended in 10µl of dH_2O.

2.2.9 Restriction enzyme digestion of plasmid DNA

Restriction digests were carried out either in small scale for investigative purposes or in large scale for the linearization of plasmids intended for transfections, preparation of probes for southern blotting or for cloning purposes. In both cases, the total volume of enzyme used did not exceed 0.1 volumes, the corresponding buffer was always used at 1x and incubations were carried out at the temperature recommended by the manufacturer. For small-scale digests, the total volume did not exceed 20µl with 1 - 5µl DNA. Large-scale digests were carried out in a total volume of 100µl with approximately 10-20µl of DNA. Purification was carried out by running the digest in an LMP 1%(w/v) agarose gel, excising the band of interest and purifying the DNA fragment from the melted gel. The digest was analysed using a normal 0.8%($^{w}/_{v}$) agarose gel and purifying the fragment by Gene-Clean (Strata-tech Scientific, Luton, UK) after excision of the band. Gene-Clean purification was carried out according to the manufacturer's instructions.

2.2.10 Blunt – ending of plasmid DNA overhangs

3' overhangs were blunt ended by a 1-hour incubation at 37°C of the DNA fragment of interest with 15 units of T4 DNA polymerase (Promega, Southampton, UK) and 1µl of 25mM dNTP stock. If further enzyme processing was required, the T4 Polymerase was heat inactivated at 80°C for 30 minutes and subsequently cooled on ice for 20 minutes. Finally, the DNA was purified by phenol/chloroform, precipitated using ethanol and re-suspended in 10µl of H_2O.

2.2.11 Ligation of plasmid DNA

Ligations were carried out in a total volume of 25µl, with 1x T4 Ligase buffer and 3 units of T4 DNA Ligase. The volumes of insert and vector used were 8 and 2µl respectively. Reactions were carried out in a cycle of 2-minute incubation at 16°C followed by another 2-minute incubation at 36°C. The total number of cycles used per ligation was 21. After ligation was complete the mixture was transformed in *E. Coli* XL1-Blue competent cells.

2.2.12 Small scale viral DNA extraction

A single well of a six well plate infected with the virus in question was harvested and the cells were spun at 3,000 rpm for 10 minutes. The pellet was re-suspended in 200µl of 50mM TES. 200µl of 10%($^{w}/_{v}$) SDS and 200µl of 100mM β-mercaptoethanol were then added. The mixture was chilled on ice for 30 minutes and incubated overnight at 55°C with 10µl of 20µg/ml Proteinase K/10mM $CaCl_2$ solution. DNA was extracted using phenol/chloroform/IAA (24:1), precipitated with ethanol/0.5 M ammonium acetate, re-suspended in 50µl of ddH_2O.

2.2.13 Large scale viral DNA extraction

The volume of virally infected cultures required for sufficient viral DNA extraction depends on how disabled the virus in question is. In the case of 1764/27^{-}/4^{-} viruses, twelve 175cm^2 plates were required for obtaining a moderate amount of viral DNA. Once the cells are harvested, they were transferred in 250ml Beckman centrifuge tubes and spun at 12,000 rpm for two hours at 5°C. The pellet was re-suspended in 10mls of Proteinase K Buffer/Proteinase K (10mM Tris pH 8.0, 5mM EDTA, 0.5%(w/v) SDS, 50µg/ml Proteinase K). Cell lysis was carried out in an orbital shaker, at 37°C overnight. The next day, 10mls of dH_2O were added to the clear lysate followed by an equal volume of Phenol/Chloroform/IAA (25:24:1). The lysate was mixed by gentle inversion for ten minutes, divided into Beckman polyallomer centrifuge tubes and spun at 15,000 rpm for 30 minutes. The top layer from each tube was removed, without disturbing the white interface appearing between the two layers and pooled in a 50ml falcon tube. An equal volume of phenol/chloroform/IAA was added again and the spinning steps were repeated until no interface appeared between the two layers. When the layers were pooled for the last time, an equal volume of chloroform/IAA was added and the lysate was spun at 2,000 rpm for ten minutes. The top layer was transferred to a new falcon tube, precipitated with ethanol and air-dried overnight at room temperature. The DNA was finally re-suspended in 100µl of dH_2O and stored at -20°C.

2.2.14 Southern blot analysis of viral genomes

Southern blots were performed on digested viral DNA of recombinant and control viruses in order to confirm that homologous recombination has produced the right genetic outcome. 10µl of purified viral DNA was digested overnight with the required enzymes, in a total volume of 50µl. Usually, a plasmid control digest was also included for correct insert size identification. 0.1µg of plasmid DNA was digested in a total volume of 20µl. Viral and plasmid DNA samples were analysed in a 1.0% TAE agarose gel.

2.2.15 Transfer of DNA onto a nitrocellulose membrane

After the bands of interest were well separated, the gel was placed on a UV trans-illuminator for 2 minutes and then denatured by soaking the gel in a large volume of Denaturing Solution (1.5M NaCl, 0.5M NaOH) for 45 minutes, rinsed in dH_2O and transferred in Neutralising Solution (2M NaCl, 1M Tris pH 5.5) for 45 minutes. After a wash with 2x SSC, the DNA was placed upside down on a plastic support covered with two layers of 3MM Whatman filter paper. This was used as a wick placed in a reservoir of 20x SSC. A piece of Hybond-N^+ membrane, was carefully placed on the gel. Any trapped bubbles were removed and 10 pieces of 3MM Whatman paper, pre-soaked in 20x SSC, were placed on top of the nitrocellulose membrane. Strips of paraffin film were used to separate the wick from the filter paper. A stack of paper towels and a weight of approximately 250g were placed on top of the filter paper. The DNA was transferred onto the nitrocellulose by capillary action, overnight, with 20x SSC as the carrier. The following day, the nitrocellulose membrane was rinsed in 6x SSC and air-dried at room temperature for 20 minutes. Finally, the DNA was the cross-linked to the membrane using a UV Stratalinker *2400*.

2.2.16 Radiolabelling of plasmid DNA probes

1µg of the plasmid DNA, containing the probe sequence, was digested overnight. Radio labelling was carried out using the Ready-To-Go™ DNA labelling Beads (-dCTP) kit. Probes were excised from a normal agarose gel and purified using the Gene Clean Kit in a total volume of 20µl. 1µl of purified probe DNA was

analysed on a gel to determine the amount required for the radio labelling reaction. The DNA was denatured by a 2-minute incubation at 90°C and immediately cooled on ice for 2 minutes. In one microcentrifuge tube containing the Reaction Mix Beads, the following were added: 1μg of denatured DNA (probe), 50μCi [α-^{32}P]dCTP (3000Ci/nmol) and dH_2O to a total volume of 50μl. The content were thoroughly mixed to ensure that the beads were fully dissolved and the mixture was incubated at 37°C for 1 hour. Unincorporated nucleotides were removed by filtering the reaction mixture through a Sephadex column (G25 to G50) using a 2cm x 1cm bed (height x diameter). The radioactive probe was again denatured as previously and added to the hybridisation solution.

2.2.17 Hybridisation

Cross-linked nitrocellulose membranes were incubated at 65°C for 4 hours with 30ml of Pre-hybridisation Solution (6x SSC, 5x Denhardt's reagent, 0.5%(w/v) SDS and 100μg/ml of denatured hearing sperm DNA in $dH2_O$). The pre-hybridisation solution was replenished but only to a reduced volume of 5ml. The denatured probe was added and hybridisation was carried out overnight at 65°C. The membrane was then washed twice for 15 minutes in 2x SSC/0.1%(w/v) SDS and once in 0.5x SSC/0.1%(w/v) SDS. The membrane was then wrapped in cling film and exposed to X-ray film at -70°C.

2.3 Cell Culture

2.3.1 Storage and recovery of cell stocks

Cells from a 175cm^2 flask were trypsinised and re-suspended in serum free media, pelleted by centrifugation at 1,500 rpm for 10 minutes and then re-suspended in 10ml of freezing solution (8%(v/v) DMSO, 30%(v/v) FCS, 68%(v/v) DMEM). Cells were stored overnight at -70°C at 1.5ml aliquots which were then transferred to liquid nitrogen for permanent storage. Each aliquot was quickly defrosted and re-suspended in 10ml of FGM. Cells were pelleted by centrifugation at 1,500 rpm for 10 minutes. The supernatant was discarded and the cells were transferred into a 25cm^2 flask containing 5mls of the appropriate, pre-warmed media.

2.3.2 Counting of cells

Counting of suspended cells was performed using the haemocytometer. A haemocytometer contains two chambers. When filled and coverslipped, each chamber contains a total volume of $9x10^{-3}$ ml. Each chamber is ruled into nine major squares. Thus, when coverslipped, the volume of each square is $0.1mm^3$ or $1x10^{-4}$ ml. Two samples of the same volume were removed from the same preparation. Cell suspensions were well shaken before the removal of samples in order to ensure accurate sampling. Using a pipette, each sample was delivered to each chamber of the haemocytometer by capillary action. It was ensured that the samples did not overfill the chamber. The total number of cells present in five of the nine squares in each of the two chambers of the haemocytometer were counted for a total of ten squares. Cells that overlapped the border on two sides of the square were included in the cell count and not counted on the other two sides. The number of cells in a total of ten chambers was counted to give the number of cells in $1x10^{-3}$ ml ($1.0x10^{-4}$ ml per square x 10 squares = 10^{-3} ml). The total number of cells was then multiplied by 1000 to give the number of cells per millimetre in the sample counted. After use the haemocytometer was cleaned with 70% Ethanol and dried with lens paper. Each well of suspended cells was counted four times to ensure accuracy.

2.3.3 Routine cell passage

Cells were grown on a variety of tissue culture plastics: $25cm^2$, $80cm^2$ and $175cm^2$ flasks and $500cm^2$ plates. For passaging purposes, cells were trypsinised when they reached a 80-90% confluence. After removal of the growth media, the cell monolayer was briefly rinsed with Hank's balanced salt Solution (HBSS). Cells were detached with trypsin/versene solution (1:10). Cells were re-suspended in fresh media and new flasks were seeded at a ratio of 1:6 for MAM49 and 1:10 for BHK cells.

2.3.4 Transient transfection of plasmid DNA

Transient transfections were carried out on BHK or BHK-based cell lines. Cells, grown on 6-well plates, were transfected after reaching a 70 – 80% confluence. The procedure followed was based on the standard calcium phosphate transfection method. 5-10μg of super coiled plasmid DNA was used per transfection. The following solutions were required:

HEBES Transfection Buffer	• 140mM NaCl • 5mM KCl • 0.7mM Na_2HPO_4 • 5.5mM D-glucose • 20mM Hepes • pH was adjusted to 7.05 with NaOH The buffer was filter-sterilised with a 0.2μm filter and stored at 4°C.
Tube A	31μl 2M $CaCl_2$ 10μg plasmid DNA 20μg Herring sperm DNA (phenol extracted)
Tube B	400ml HEBES Transfection buffer
Shocking Solution	25% (v/v) DMSO in HEBES transfection buffer

The contents of tube A were thoroughly mixed and added to tube B in a slow and steady flow with simultaneous gentle vortexing. The DNA was allowed to precipitate at room temperature for 20-40 minutes. The media was removed from the cells and the mixture containing the precipitated DNA was added in a drop wise manner. Following 20-40 minute incubation at 37°C, 1ml of full growth media was added. Cells were returned to 37°C for another 4-6 hours. The media was then removed and the monolayer was washed twice with 2mls of growth media. 1ml of shocking solution in HEBES transfection buffer was added to the cells, which were then left without shaking at room temperature, for exactly 150 seconds. The shocking solution was quickly removed and the cell monolayer was immediately washed twice with 2mls of growth media. Finally, 2mls of full growth media, without antibiotic selection, were added and the cells were incubated for a further 24 - 48 hours at 37°C under a 5% CO_2, humidified atmosphere.

2.3.5 Detection of GFP expression

Cells expressing GFP could be directly visualised using an inverted fluorescent microscope at a wavelength of 520nm.

2.3.6 Detection of β-gal expression

Full growth media from the monolayer in a well of a 6-well plate was removed and cells were washed twice with 2mls of 0.1M PBS. They were then fixed by a 10-minute incubation with 0.1M PBS/0.05% (v/v) glutaraldehyde. Cells were again washed twice with 2mls of 0.1M PBS and incubated at 37°C for 1-16 hours with 3mls of X-Gal stain (5mM $K_3Fe(CN)_6$, 5mM $K_4Fe(CN)_6.6H2O$, 1mM $MgCl_2$, 150μg/ml X-Gal in DMSO made up in 0.1M PBS). If storage was required then the stain was removed and the cell monolayer was overlaid with 2mls of 70% glycerol in dH_2O. If the staining was carried out for the purposes of plaque picking, the cells were not fixed prior to staining.

2.4 Viral vector construction and propagation

2.4.1 Homologous recombination into the HSV1 genome

Insertion of expression cassettes in the HSV1 genome by homologous recombination was achieved by transfections on the appropriate cell line. 10-20 μg of purified viral DNA was added in tube A. The following mixing and vortexing steps were carried out in a more cautious manner. The transfection mix was left for 7 hours prior to DMSO shocking. Transfections were left for 4-6 days until complete cytopathic effect (CPE) had occurred. As the viruses used and produced in this study had a deactivated VP16 gene, the growth media had to be supplemented with 3mM hexamethylene bisacetamide (HMBA), which can induce immediate early gene transcription (McFarlane et al 1992). Once complete CPE had occurred the transfections were harvested and freeze-thawed once. A 100μl aliquot of the transfection was serially diluted and the efficiency of the recombination was assessed by the presence of viral plaques that had the new phenotype encoded by the inserted gene.

2.4.2 Purification of recombinant virus by plaque selection

After titration of the transfections, cells were not fixed. Instead they were stained for the presence of β-galactosidase (β-gal). The presence of β-gal was monitored carefully and blue viral plaques were picked as soon as the colour started to develop. This was due to toxicity of the staining procedure. Recombinant plaques were picked from the monolayer at a volume of 3μl, using a P20 Gilson micropipette. Each plaque was thoroughly re-suspended in 100μl of SFM, freeze-thawed and plated out in two wells of a 6-well plate at 40 and 60μl. The cells, 70% confluent, were overlaid with 500μl of SFM prior to infection and once the virus was added they were incubated at 37°C for two days. In the case of a highly disabled virus, incubation was allowed to proceed for four days. This procedure of picking and infecting was carried out until all plaques had the recombinant phenotype. When pure, each plaque was used to infect a single well of a six well plate. When CPE was observed, the virally infected cells were harvested, stored at -80°C and one aliquot was used to titre the stock on both complementing and non-complementing cells. This was done in order to make sure that there were no reversion mutants. In addition to titration, viral DNA from recombinant plaques was also extracted in a small-scale preparation and analysed by southern blotting for the presence of the gene inserted.

2.4.3 Production of high titre HSV-1 stocks

Minimally disabled viruses, such as 1764, were grown in Corning 850cm^2 roller bottles. BHK cells were seeded in 100 ml of full growth media. Roller bottles were gassed with 5%CO_2 through a filtered gas line and incubated at 32°C in a rotated platform at 0.5rpm. When cells reached 90% confluence they were infected at an MOI of 0.01 (1x 10^6 pfu/ml of virus per roller bottle). in a total volume of 100ml FGM supplemented with 3mM HMBA. Roller bottles were then incubated at 32°C/5% CO_2 for a further two to three days, until full CPE had occurred. Cells were detached from the walls of the roller bottles by vigorous shaking. The cell suspension was the frozen at -80°C. Ten roller bottles were infected for each batch preparation.

Disabled viruses that had essential and immediate early genes deleted or inactivated were grown in large 500cm^2 tissue culture plates. Ten large plates were used for each high titre viral stock. Complementing cells 27/12/M:4 (Thomas *et al.*, 1999) were seeded in ten large plates in a total volume of 50mls of FGM per plate. Cells were incubated at 37°C/5% CO_2 until they reached 90% confluence. They were then infected at an MOI of 0.05 (3x 10^6 pfu/ml of virus per 500cm^2 plate). The virus was used in a total volume of 50ml FGM supplemented with 3mM HMBA. Plates were returned to 37°C/5% CO_2 until complete CPE had occurred. They were then frozen at -80°C.

The cell suspension was then thawed and centrifuged at 3500rpm for 45 minutes at 4°C in order to remove any cell debris. The supernatant was then separated from the cell pellet and sequentially filtered through a 5μm and 0.45μm filter. The virus was then pelleted by a 2-hour, 12000rpm centrifugation at 4°C. The supernatant was then discarded and the viral pellet was re-suspended in fresh, ice cold DMEM. The final volume of the viral stock depended on the level of disablement as well as the quality of each preparation. Generally, minimally disabled viruses were re-suspended in 1-3mls per 10 roller bottle stock. Highly disabled viruses were re-suspended in 300-500ml per 10 500cm^2 plates. In each case, the viral pellet was sonicated five times in 10-second bursts. The stock was chilled on ice for 20 seconds in between sonication steps. Once the suspension was homogeneous, the virus was stored in liquid nitrogen in 20-50μl aliquots. One aliquot was freeze-thawed and used to determine the viral titre.

2.4.4 Establishing the viral titre of HSV-1 stocks

Six-well plates were seeded with the appropriate cells and incubated at 37°C until confluence had reached 80-90%. The FGM was then removed and 500μl of DMEM, also referred to as Serum Free Medium (SFM), were aliquoted in each well. The virus was then added to the monolayer of each well in a 1:10 serial dilution in SFM, progressing from 10^{-1} down to 10^{-6}. Following a one hour incubation at 37°C, each well was overlaid with 1.5ml of FGM/3mM HMBA containing 1.6% carboxymethyl cellulose (CMC) in 1:2 ratio. Plates were then

incubated at 37°C for a further two days. In order to calculate the viral titre, as plaque forming units per ml of stock (pfu/ml), cells were either fixed and stained for *lacZ* expression, or examined under fluorescence for the expression of Green Fluorescent Protein (GFP).

2.5 Protein isolation and analysis

2.5.1 Concentration of secreted proteins from cultured cells

$1x10^6$ cells in a 36mm well were washed twice with 0.1M PB. 500ml of DMEM were aliquoted in each well and the cells were returned to a 37°C/5%CO_2 incubator. At the desired time point, the DMEM was collected and centrifuged at 2,000rpm for 5 minutes to remove any cells present. The supernatant was then transferred to a 500µl microcon column (G-10000, Green) and spun at 11,500g for 25 minutes at room temperature. The microcon columns used for the experiments described in this study had a molecular weight cut-off of 10Kda as the proteins of interest were in the region of 12-14Kda. The total volume of the concentrated sample was 10µl. An equal volume of protein sample buffer [5% (v/v) b-mercaptoethanol, 50mM Tris-HCl pH 8.0, 2% (w/v) SDS, 6% (v/v) glycerol and 0.005% (w/v) bromophenol blue] was added. Samples were placed on ice immediately and then denatured by heating to 95°C for 4 minutes. They were then cooled on ice for 2 minutes and either loaded onto a SDS-polyacrylamide gel or stored at -20°C.

2.5.2 Extraction of intracellular proteins from cultured cells

$1x10^6$ cells in a 36mm plate were washed twice with 0.1M PB and harvested in 100µl of protein sample buffer. Harvested cells were placed on ice immediately and then denatured by heating to 95°C for 4 minutes. Samples were then cooled on ice for 2 minutes and either loaded onto a SDS-polyacrylamide gel or stored at -20°C.

2.5.3 SDS – polyacrylamide gel electrophoresis

Protein samples were heated to 95°C for 4 minutes and then immediately cooled on ice for 2 minutes. In a SDS-PAGE gel 20µl of each sample and 5µl of coloured molecular weight protein standards (rainbow markers) were loaded in appropriate spaced lanes. Gels were prepared and run in a vertical electrophoresis system. The consistency of the stacking and resolving SDS-PAGE gels is shown below.

5% Stacking gel	• [1]*Bis-Acrylamide (30:1.5)	1.6ml
	• 4x Stacking gel buffer	2.6ml
	• 10% (w/v) APS	100µl
	• **TEMED	5µl
	• ddH_2O to a final volume of	10.55ml
15% Resolving Gel	• Bis-Acrylamide (30:1.5)	
	• 4x Stacking gel buffer	15.75ml
	• 10% (w/v) APS	7.9ml
	• TEMED	150µl
	• ddH_2O to a final volume of	9µl
		32.5ml

- 4x Stacking gel buffer: 0.5M Tris base and 0.01M SDS, pH 6.8
- 4x Resolving gel buffer: 1.5M Tris base and 0.01M SDS, pH 8.8

Protein separation was achieved by applying a constant current of 30-40mA/gel with variable voltage, in 1x running buffer (25mM Tris, 250mM glycine and 0.1% (w/v) SDS, pH 8.3). Gels were run until the bands of interest were fully separated, as judged by the migration pattern of the coloured molecular weight markers.

2.5.4 Equalisation of protein loading

Duplicate protein samples were analysed on a separate SDS-PAGE gel. This gel was placed in Coomassie stain solution [2% (w/v) Coomassie Brilliant blue R250, 50% (v/v) Methanol, 50% (v/v) glacial acetic acid] for 1 hour at room temperature with continual agitation. The gel was then transferred to de-staining solution [10% (v/v) glacial acetic acid, 30% (v/v) Methanol] for the removal of any non-protein bound stain. The de-staining solution was replaced twice until no background staining was visible.

[1] *Bis: N,N'-Methylene-bis-acrylamide, **TEMED: NNNN-tetraethylethalinediamine.

2.5.5 Western blotting

Separated protein bands on an SDS-PAGE gel were transferred onto a nitrocellulose membrane (Hybond-C) using a wet-transfer method based on the technique outlined in (Towbin, et al., 1979). Briefly, the SDS-PAGE gel, the nitrocellulose membrane and four sheets of 3MM Whatman paper were pre-soaked in transfer buffer (50mM Tris, 380mM glycine, 0.1% (w/v) SDS and 20% (v/v) methanol). The gel and the nitrocellulose membrane were then sandwiched between the filter paper in a Trans-Blot™ Cell (BioRad) according to the manufacturer's instructions. Protein band transfer onto the nitrocellulose membrane was carried out overnight at 4°C at 200mA. Protein bands were visualised by staining the membrane with 0.5% (w/v) Ponceau S in 1% (v/v) acetic acid for 30 minutes. Excess stain was removed by washing the membrane in several changes of 0.1M PB.

2.5.6 Immunodetection of proteins on nitrocellulose membranes

Following transfer, membranes were washed in 0.1M PB and then blocked by incubating them in 5% (w/v) skimmed milk made up in 0.1M PBS for 1 hour, at room temperature, with shaking. The membrane was then incubated with the primary antibody, diluted in 3% (w/v) skimmed milk, for 1-2 hours at room temperature, with vigorous shaking. All the antibodies used in this thesis, as well as their working dilutions are listed in section 2.1.6 (page 84). Any unbound antibody was removed from the membrane by three washes for 10 minutes each with PBST [0.1M PBS, 0.1% (v/v) Tween-20] at room temperature with constant shaking. Washed membranes were then incubated in the appropriate anti-IgG horseradish peroxidase (HRP) conjugated secondary antibody, diluted in 3% (w/v) skimmed milk made up in 0.1M PBS for 1 hour, at room temperature, with vigorous shaking. Any unbound antibody was removed from the membrane by three washes, for 10 minutes each with PBST, at room temperature with shaking. The bound HRP was visualised using the Enhanced Chemiluminescence system (ECL™) according to the manufacturer's instructions. The membrane was then exposed to X-ray film to capture the resulting light emissions. Exposure times varied between 10 seconds and 15 minutes, according to the strength of the signal.

2.5.7 Enzyme linked immunosorbent assay for NT3 detection

Each well of a 96-well Nunc-MaxiSorp plate was incubated overnight at 4°C with 100µl of anti-Human NT-3 polyclonal antibody in Coating Buffer pH 9.7 (0.025M Sodium Carbonate, 0.025M Sodium Bicarbonate). After washing the wells 5 times with TBST buffer (20mM Tris-HCl pH 7.6, 150mM NaCl, 0.05%($^{v}/_{v}$) Tween-20), using a plate washer, non-specific sites were blocked with 1x Blocking buffer for 1 hour. The TBST washing step was repeated after every incubation step. A standard curve was prepared by serially diluting the NT-3 standard from 4.7 to 300pg/ml in 1x sample buffer. The samples, i.e. the transfection supernatants, were added undiluted. The plate was then incubated for 6 hours at 4°C at 500 rpm. Once the incubation was complete, the anti-NT-3 Ab was added and the plate was incubated overnight at 4°C, without shaking. The next day the samples were incubated at room temperature with anti-mouse IgG HRP conjugate for one hour at 500rpm. Colour reaction was initiated with the addition of TMB substrate and it was stopped after 15 minutes with the addition of 100µl/well of 1M Phosphoric Acid. The sample and standard UV absorbance values were measured at 450nm and a standard curve was generated. The experimental values obtained were considered accurate if the standard curve generated had an r^2 value greater than 0.98.

2.6 Primary cultures

2.6.1 Isolation of E14 rat embryonic dorsal root ganglia (DRG)

An E14 pregnant Lewis rat was terminated via CO_2 administration. The rat was transported to a laminar flow hood and subsequent steps were carried out in sterile conditions. The abdominal area was sprayed with 70%(v/v) Ethanol and an incision was made along the lower abdomen. The chain of pods containing the embryos was removed and placed in a Petri dish containing sterile 0.1M PBS. Each embryo was removed from the amniotic sack and placed in a new Petri dish also containing sterile 0.1M PBS. The tail and head were removed and an incision was made along the length of the spinal column. The spinal cord was then removed with the dorsal root ganglia still attached. Each DRG was then

snipped off and cleaned of any membrane surrounding it. The cleaned DRGs were pooled in ice-cold Leibovitch medium (L-15; Sigma) and kept on ice under sterile conditions. DRGs were plated out in supplemented media as quickly as possible.

2.6.2 Setting of DRGs in collagen matrices

All the steps listed in this procedure were carried out under sterile conditions. In a 15 ml falcon tube, 8mls of chilled Vitrogen Collagen were thoroughly mixed with 1ml of 10x PBS. 1ml of 0.1M NaOH was then added and the contents we again mixed. The pH was then adjusted to 7.4 ± 0.2 by the addition of either 0.1M HCl or 0.1M NaOH accordingly. The now neutralised and isotonic Vitrogen Collagen solution was stored at 4°C for a maximum of 3 hours. A 50µl drop was placed in a tissue culture dish and a DRG explant was gently embedded in it. Fibrillogenesis was initiated by a 1-hour incubation at 37°C in the absence of CO_2. Once the collagen had fully set, explants were flooded with chemically defined medium (DMEM/F12 (3:1) supplemented with 200µg/ml transferrin, 200µM putrescine, 40nM progesterone, 60nM selenium, 10µg/ml insulin and 0.01% (w/v) albumin).

2.7 *In vivo* gene delivery - Animal surgery

2.7.1 Anaesthesia

Female LEWIS Rats, as close to 200g as possible, were initially anaesthetised by a mixture of 1.5% Oxygen, 3% Nitrous oxide and 4% Halothane (Fluothane, ICI) administered within an anaesthetic chamber. Once deep anaesthesia was established, animals were transferred into the operating theatre and placed on an electrically heated pad. The anaesthetic mixture was adjusted to 1.5 – 2% Halothane and was administered by means of a scavenging face/nose mask. Post-operatively, the animals were kept in a clean cage and monitored every hour for the first 6 hours and twice a day after the first day, until stable.

2.7.2 Animal Preparation

Prior to any procedure, animal fur was shaved using veterinary clippers. The exposed areas were then thoroughly cleaned by means of a 70% Ethanol solution. The animal was then covered with a sterile surgical cloth, exposing only the area of interest.

2.7.3 Injections into the spinal cord

A 25µl Hamilton syringe fitted with a fine needle (outer Ø 300 µm) was used to administer the vector at a 1.5 mm depth into the uninjured spinal cord or within the lesion site in CST lesioned animals. A World Precision Instruments microinjection controller (Micro4 Smart Controller, WPI, Europe) in combination with a type II ultra-micropump (Ultra-Micropump II), in order to administer the vector at a steady rate. Delivery of the vector in these experiments was done by convection. Convection relies upon creating a pressure gradient that forces the viral particles into the solid neural tissue. The delivered particles then diffuse into the tissue surrounding the injection site (Chen *et al.*, 2005). Viral suspensions were injected at a rate of 0.5 µl/min using a micro-pump. For the purposes on this thesis, spinal cord delivery was carried out either at the lumbar level (L3-4) or at the cervical level for (C6-7). The needle was slowly removed, the dura, spine muscles and the overlaying skin was then repaired with Michel clips.

2.7.4 Injections in the cerebellum

The co-ordinates for injecting the motor cortex were: Rostral-Caudal 11.5 mm, Medial-Lateral 1.5 mm, Dorsal-Ventral 3.0 mm.

2.7.5 Foot – pad injections

Three-week-old female BALB/c or SCID mice were inoculated via right footpad injection using an insulin needle. Virus of approximately $1x10^8$ pfu/ml was inoculated at a volume of 20-25µl. BALB/c mice remained sedated under 5% Halothane. In the case of SCID mice this was not possible and injections had to be carried out in a sterile hood without anaesthesia.

2.7.6 Mouse DRG extraction

At the appropriate time point, mice were sacrificed. DRGs from the lumbar spine (L1 to L6) and ipsilateral to the inoculated side were dissected and fixed for 1 hour at 4°C with 2% Paraformaldehyde (PFA) in 0.2M Phosphate Buffer pH7.4. After incubation they were washed twice with 0.1M PBS and examined for the expression GFP. They were then stained with X-gal stain and incubated at 37°C overnight for assessing β-gal activity.

2.7.7 Perfusion of animals

The animals were terminally anaesthetised with CO_2. The heart was exposed, and after an incision of the pericardium a blunt needle was inserted in the left ventricle. 4% PFA (in 0.2M Phosphate Buffer pH 7.4) was pumped through the heart using a peristaltic pump at a rate of approximately 30 rpm. In the cases were tetramethylbenzidine processing of tissue was required, animals were fixed with a solution of 1% (w/v) PFA/1.25% (v/v) Glutaraldehyde. Once perfusion was completed, the animals were dissected and the tissue of interest was collected. Tissue was post-fixed in 4% PFA for 1 hour on ice and washed twice with 0.1M PBS.

2.7.8 Processing of brain and spinal cords

Extracted tissue (spinal cords and brains), were cryo-protected by overnight incubation in 30%($^{w}/_{v}$) Sucrose at 4°C. The next day they were sectioned to the desired thickness, usually 40μm for spinal cords and 70μm for brains. Free-floating sections were further processed and they were then mounted onto slides and coverslipped for permanent keeping.

2.7.9 Processing of optic nerves

Extracted optic nerves were kept in fixative overnight at 4°C before being dehydrated and embedded in low melting point polyester wax (Aldrich). Longitudinal sections were cut at 10μm on cryostat, washed in PBS, incubated for 15 min with 0.3% (v/v) H_2O_2 in PBS to remove endogenous peroxidase

activity, washed in PBS, exposed to blocking serum, (5% normal rabbit serum) and 0.3% TritonX-100 in PBS and then incubated with goat anti-CTB (List Biological, Campbell, California) 1:80,000 in blocking serum for 2 days at 4°C. They were then washed in PBS, incubated for 2hr with rabbit anti-goat IgG 1:200 in PBS, washed in PBS and treated for 2hrs with the avidin-biotin peroxidase complex (ELITE Kit, Vector, USA, 1:200 in PBS).

2.8 Immunological and histological techniques

2.8.1 Immunostaining on tissue sections

This method was used for SCG10 and GFAP immunolabelling (Chapter 5). Cryostat sections were dried overnight and their boundaries marked with a grease pen. The following day they were rinsed three times with 0.1M TBS for 10 minutes each time. They were then incubated in 0.1% (v/v) Hydrogen Peroxide in 0.1M TBS for 15 minutes to quench endogenous peroxidases. After washing twice for 5 minutes in 0.1M TBS, sections were incubated in Blocking Buffer (10% (v/v) Goat Serum, 5% (w/v) Bovine Serum Albumin in 0.1M TBS) for 30 minutes at room temperature. The blocking buffer was removed and sections were incubated with the primary antibody, diluted in 0.1M TBS, at 4°C, overnight. Sections were washed three times for 10 minutes with washing buffer (0.1M TBS/0.3% (v/v) Triton x100) and incubated in the biotinylated secondary antibody, diluted also in washing buffer, for 2 hours at room temperature. Sections were then washed twice for 10 minutes in washing buffer and twice for 10 minutes in 0.1M TBS. They were then incubated in ABC complex/0.1M TBS at a 1:200 dilution, for 1 hour at room temperature. After washing twice for 5 minutes in 0.1M TBS, sections were incubated in 0.005% (w/v) DAB with 0.01% (v/v) Hydrogen Peroxide in 0.05M TB for 5 minutes at room temperature. Once the reaction was completed, sections were washed 3 times with 0.05M TB, rinsed with dH_2O and left to dry overnight.

2.8.2 Immunofluorescence on tissue sections

Immunofluorescence was carried out primarily on free-floating sections as this allowed better penetration of the antibodies used. Freezing microtome sections were washed three times for 10 minutes with washing buffer (2% (w/v) Skimmed milk, 0.05% (v/v) Tween-20 in 0.1M TBS). Non-specific sites were blocked by a one hour incubation with Blocking Buffer (1% (w/v) BSA, 10% Goat serum, 0.25% (v/v) Triton x100 in 0.1M TBS), at room temperature on a rocking platform. Sections were incubated in primary antibody diluted in blocking buffer, at 4°C overnight. After washing three times for 10 minutes with washing buffer, sections were incubated with the secondary antibody for 2 hours at room temperature with gentle rocking. The streptavidine fluophore secondary antibody was diluted in blocking buffer at a 1:200 dilution. The New England Nuclear (NEN) Tyramide Signal Amplification (TSA™) Kit was used to enhance the fluorescent signal. Sections were washed three times for 10 minutes in TNT washing buffer and incubated for 1 hour at room temperature in streptavidin-HRP diluted in blocking buffer (1:100). Again, sections were washed three times for 10 minutes at room temperature with TNT washing buffer. They were then incubated in Fluorophore Tyramide, diluted 1:50 in amplification diluent for a maximum of 10 minutes at room temperature. Sections were washed again in TNT washing buffer, three times for 10 minutes. Finally, sections were mounted onto gelatinised slides and coverslipped using PBS/glycerol 1:9 with 2.5% (v/v) 1,4-diazabicyclo[2.2.2]octane (DABCO).

2.8.3 Immunofluorescence E14 rat DRG explants

DRG explants were washed twice with 0.1M PBS. They were then fixed with 4% (w/v) Paraformaldehyde in 0.1M PB for 15 minutes at room temperature without shaking then washed three times with 0.1M PBS and permeabilised by a 5 minute incubation in 0.1M PBS/0.1% (v/v) Triton x100. After a brief wash with 0.1M PBS they were incubated in 0.1M PBS/30% (v/v) Goat serum for 30 minutes to block any non-specific sites and then incubated for 1 hour at room temperature with the mouse TUJ1 anti neuronal – specific tubulin, in a final dilution of 1:5000. TUJ1 was diluted in 0.1M PBS/10% (v/v) Goat serum. The primary antibody was removed and explants were washed three times with 0.1M PBS.

Then, they were incubated for 2 hours in a light protected container with anti mouse IgG2A rhodamine conjugated antibody. The secondary antibody was used at a 1:1000 dilution in 0.1M PBS/10% (v/v) Goat serum. Explants were then washed in 0.1M PBS and photographed the same day.

2.8.4 Tracing of neurons by biotin dextran (BDA) processing

BDA is an anterograde tracer and it was injected in motor cortex or the red nucleus for labelling of corticospinal or rubrospinal tract neurons respectively (Chapter 4 and 5 respectively). Processing was carried out on free-floating sections based on a well known method (Herzog and Brosamle, 1997). Sections were washed three times for 10 minutes in 0.05M TBST (0.05M TBS /0.5% (v/v) Triton x100). They were then incubated in 0.3% (v/v) Hydrogen Peroxide to quench endogenous peroxidases. After washing them twice for 10 minutes in 0.05M TBST and incubated overnight at 4°C in ABC solution. The ABC solution was used in a final dilution of 1:200 and consisted of reagents A and B (50:50). The following day sections were washed twice in 0.05M TBST and twice in 0.05M TB, for 10 minutes each time. They were then rinsed in 0.4% (w/v) ammonium nickel sulphate for 10 minutes. They were then incubated in 0.0015% (w/v) DAB made up in 0.15M TB, pH 7.4. To this, hydrogen peroxide was added to a final volume of 0.01%. The reaction was allowed to proceed until the desired colour intensity was achieved. Finally, sections were washed twice in 0.05M TB for 10 minutes, mounted onto gelatinised slides and processed as in section 2.8.9.

2.8.5 Tracing of neurons by cholera toxin - b (CTB)

Sections were rinsed in dH_2O and placed in reaction solution (3.4mM Sodium Nitroferricyanide, 23.4mM Sodium Acetate pH 3.3, 0.2mM Tetramethylbenzidine) for 20 minutes. Hydrogen peroxide was added at a final volume of 0.01% (v/v) and the reaction was allowed to proceed for 5 minutes on ice. The solutions were then discarded and the above step was repeated until the reaction solution had been replaced six times. Sections were then rinsed twice in 100mM Sodium Acetate buffer pH 3.3 for 5 minutes each time. Finally, they were mounted onto gelatinised slides and further processed as in section 2.8.10.

2.8.6 Detection of β-Galactosidase (β-Gal) in DRG

Extracted DRG were placed in ice cold 0.1M PBS. These were then fixed with 4%($^{w}/_{v}$) Paraformaldehyde in 0.1M phosphate buffer pH 7.6. After a 1 hour incubation on ice, DRGs were washed twice in 0.1M PBS for 15 minutes each wash. The DRGs were then incubated in 100µl of DRG X-Gal stain made up in 0.1M PBS (5mM $K_3Fe(CN)_6$, 5mM $K_4Fe(CN)_6.6H_2O$, 1mM $MgCl_2$, 0.02% Sodium Deoxycholate, 0.02% NP-40 and 40mg/ml X-Gal in DMSO) overnight at 37°C. The X-Gal solution was the removed and DRGs were placed in a 70%($^{v}/_{v}$) glycerol solution and stored at 4°C prior to photography.

2.8.7 Detection of β-Gal in Retinas

Retinas were placed in ice cold 0.1M PBS and later fixed with 4%($^{w}/_{v}$) Para formaldehyde in 0.1M phosphate buffer pH 7.6 for 1 hour on ice. During that time retinas were kept attached on a silicone bed with fine steel needles. Retinas were washed twice in 0.1M PBS for 15 minutes each wash and incubated overnight at 37°C in X-gal stain. Once processing was complete, retinas were mounted onto slides and covered with a drop of glycerol. The coverslip was sealed using nail varnish and the slides were stored at 4°C prior to photography.

2.8.8 Detection of β-Gal in free-floating spinal cord & brain sections

After sectioning, slices were washed twice in 0.1M PBS and examined under fluorescent light for the expression of GFP. The presence of LacZ was examined by staining the slices with X-gal stain at 37°C. The X-gal stain used was not the same as the one used for DRG staining as the presence of detergents could damage the integrity of the slices. Instead tissue culture stain was used as described previously for viral plaque selection. β-Galactosidase presence was monitored to avoid the development of background. After blue colour development, the slices were washed with 0.1M PBS and counter-stained with Neutral red.

2.8.9 Detection of GFP expression in DRGs and tissue sections

DRGs and free-floating tissue sections were transferred onto a glass slide using a pipette tip and artists fine brush respectively. They were then coverslipped and GFP expression was visualised under UV light (520 nm) and photographed. GFP fluorescence was always recorded prior to any further processing, as it is susceptible to bleaching by detergents used in other techniques such as X-Gal staining.

2.8.10 Counter staining of sectioned tissue

Tissue sections were mounted onto gelatinised microscopic slides by immersing them into 0.2M Phosphate Buffer pH 7.4 and slowly guiding each one onto the slide using a fine tipped artist's brush. They were allowed to dry overnight at room temperature and the following day they were fully immersed into Neutral Red stain. (4mM Sodium Acetate pH 8.5, 24mM Acetic Acid pH 2.4 and 0.02%($^{w}/_{v}$) Neutral Red) until they were stained a dark red colour. Sections were dehydrated by sequentially soaking them in 70%, 90% and 100% Ethanol for 10 minutes each time. Finally, tissue fats were removed by incubating in Histoclear for 30 min. Once counter-staining was complete, cover slips were fixed on the slides using DPX mounting solution (BDH, Poole, UK).

CHAPTER 3.0

DISABLED HSV1 VECTOR BACKBONES FOR TRANSGENE DELIVERY TO THE CNS

3.1 Introduction

As reviewed in Chapter 1, its natural propensity to infect terminally differentiated cells, its neurotropism as well as its ability to be retrogradely transported to the nucleus and enter latency which can be maintained for the lifetime of the host, are the key characteristic that make HSV-1 an attractive vector for gene delivery in the injured CNS (Lilley *et al.*, 2001a; Lilley and Coffin, 2003). The vectors employed in this thesis, carry mutations that allow them to be minimally cytotoxic and expression cassettes that utilise endogenous elements from the LAT region thus maximising their ability to achieve transgene expression in the long term. These are based on constructs that were produced in our laboratory, but whose potential as a gene delivery tool to the CNS had not yet been assessed *in vivo* until the initiation of the study described here. Out of a panel of vectors with different levels of disablement that became available as the study progressed, two vectors were particularly selected. Firstly, a fully disabled vector: 1764/ICP27$^-$/P2$^-$/ICP4$^-$/pR20.5*vhs* (HSV1/P2$^-$.pR20.5*vhs*) and a slightly less disabled vector: 1764/ICP27$^-$/ICP4$^-$/pR19CMV (HSV1.pR19CMV). The construction and genetic make up of these two vectors has been published (Palmer *et al.*, 2000; Lilley *et al.*, 2001b) and is briefly reviewed in section 3.2.1 (page 126). These vectors were selected because they presented the best practical compromise between the necessary ability to transduce neuronal cell populations, while ensuring minimal levels of cytotoxicity to the host. The expression patterns achieved with these vectors are characterised in the following sections. The focus is the transduction patterns achieved following a single inoculation of these vectors in various areas of the CNS such as the spinal cord, cortex and cerebellum. This approach aimed to establish the strengths and limitations of these two vectors that may define their utility. These were established along with differences in their ability to support the expression of a transgene such as β-galactosidase or GFP, which directly relates to their potential as gene delivery tools for the injured spinal cord and the CNS in general. This is particularly important, as CNS regeneration is a complex and slow process that can last from months to years. It is therefore likely that for a gene delivery system to be successful in aiding such a process, it has to be able to sustain transgene expression in the long term.

Ultimately, the main target of the studies described in this thesis was to utilise these vectors as a platform from which to screen various factors that could have a positive impact on injured neurons of the spinal cord as well as other tracts such as the optic nerve. HSV1/P2$^-$.pR20.5*vhs* and HSV1.pR19CMV vector backbones, used in this thesis, were constructed by Dr C.E. Lilley following previous work in our laboratory. These two disabled vectors were chosen for the production of the neurotrophin expressing constructs described in the following chapters. The rationale behind their use is discussed in the sections below.

3.1.1 Reducing vector cytotoxicity

Wild type HSV1 is highly pathogenic. When HSV1 was first under investigation for its potential as a gene delivery tool, a variety of researchers attempted to establish the origin of the toxic effect that follows the inoculation of the virus into the CNS. Extensive work in our laboratory led us to conclude that the main cause of cytotoxicity is due to the production of the α proteins which takes place during the establishment of the lytic cycle. It was not due to a particular feature of the virion itself or due to the processes occurring during viral entry into the host (Johnson *et al.*, 1992).

As discussed in Chapter 1, ICP4 is the primary transactivator responsible for regulating the expression of the downstream β and γ viral genes (DeLuca *et al.*, 1985) and has been shown to be cytotoxic in many cell lines, including neuronal cells (Johnson *et al.*, 1992). It was therefore logical to suggest that deletion of ICP4 would reduce the level of expression of the remaining HSV1 genes and prevent the virus from entering a lytic life cycle (DeLuca *et al.*, 1985). Indeed, forcing the virus into latency forms the basis of the vectors utilised in our experiments. However, initial studies utilising temperature sensitive mutants suggested that the sole deletion of ICP4 aggravated rather than reduced cytotoxicity (DeLuca *et al.*, 1985). In these experiments it was shown that when ICP4 expression was blocked, the expression of the rest of the α genes was not abolished. IE genes encoding ICP0, ICP22, ICP27, ICP47 as well as ICP6, a viral protein that does not rely on ICP4 for its induction, were still produced (DeLuca *et al.*, 1985). It is therefore not surprising that fibroblasts infected with

an ICP4 deleted mutant displayed evidence of cytotoxicity, such as fragmentation of host genomic DNA and formation of cytoplasmic vesicles (Johnson *et al.*, 1992). This feature of the ICP4 deleted mutants is easily explained by the fact that ICP4 negatively regulates its own expression and that of ICP0. Consequently, deleting ICP4 alone would result in the continued expression of the remaining IE proteins which, with the exception of ICP47, are also toxic (Johnson *et al.*, 1992).

From the reported studies up to this point, a consensus was starting to emerge that deleting just ICP4 was not enough of a disablement to produce a vector that could be considered non-cytotoxic, and that it was necessary for other IE genes to be deleted as well. Evidence that support this notion came from both *in vivo* and *in vitro* experiments that employ vectors that are disabled for a combination of IE genes instead of just ICP4 mutants. Cortical or DRG neurons infected with a virus that has been deleted for the ICP4, ICP22 and ICP27 IE genes survived significantly longer (21 days p.i) than those neurons that were infected with HSV1 mutants that were individually mutated for each of these genes (4 days p.i) (Krisky *et al.*, 1998). When these vectors were injected into areas of the adult rat brain, including the frontal cortex, there was a reduced area of necrosis surrounding the injection site compared to wild-type virus injected animals (Chiocca *et al.*, 1990). Therefore, deleting a combination of IE genes has a beneficial effect in terms of reducing cytotoxicity, that can be manifested in both *in vitro* and *in vivo* paradigms.

Further experiments set out to explore which of the remaining α genes were primarily responsible for this toxic effect and determine whether there were some combinations of deletions that were more effective than others at reducing cytotoxicity. Firstly, transient transfections with plasmids encoding each of the IE genes ICP0, ICP22 or ICP27 were conducted in fibroblasts infected with an ICP4 deleted mutant virus (Johnson *et al.*, 1994). The authors realised that each of the IE genes tested (i.e. ICP0, ICP22 and ICP27), were implicated in the cytopathic effect of ICP4 deleted vectors. ICP47 and ICP6 proteins were not included in these experiments as they were not expected to be cytotoxic based on their known properties at the time (Johnson *et al.*, 1994). This finding was

confirmed when another research group demonstrated that a virus that carried a combination of ICP4, ICP22 and ICP27 deletions was markedly lacking in cytotoxicity when compared to other mutants that had these genes deleted either singly or in double combinations (ICP4 and ICP27 or ICP4 and ICP22) (Wu *et al.*, 1996; Krisky *et al.*, 1998). This combination of three deletions was unique because it allowed Vero or human embryonic lung cells (HEL) to survive for three days when infected at high MOI (MOI=10), compared to just 24 hours when infected with viruses mutated for double combinations of IE genes (Wu *et al.*, 1996). Interestingly, further examination of cellular protein expression profiles demonstrated that viruses that were deleted for ICP4 alone or ICP22 in combination with ICP27 did not cause impairment in the cell's transcriptional activity whereas there was a marked reduction in transcription when the cell was infected with a mutant that was only deleted for ICP4 (Wu *et al.*, 1996). These findings pointed to the fact that in order to produce a vector that is non-toxic for the purpose of delivering genes to the CNS, it would be necessary to silence the expression of all the remaining α genes. Whether this approach would indeed have an effect was put to the test in the work of Samaniego *et al.* who were the first to produce a mutant virus that had been deleted for all five IE genes: ICP0, ICP4, ICP22, ICP27 and ICP47. *In vitro*, Vero or HEL cells transduced by these very disabled vectors displayed the same viral protein expression patterns as cells that had not been infected with virus (mock infected) (Samaniego *et al.*, 1998). Significantly, these experiments demonstrated that highly disabled virus constructs such as the ones used by these researchers displayed such low level toxicity (if any) to host cells that they enabled the viral genome to persist in a functional state for a minimum of 28 days (Samaniego *et al.*, 1998). This finding demonstrated the significant reductions in cytotoxicity which a combined approach to deleting the IE genes can achieve.

Even though deletions in the immediate early genes themselves do achieve significant reductions in cytotoxicity, deleting each one individually is not a significantly effective strategy. Another means of causing a blanket reduction in IE genes is to delete the portion of the gene which encodes the VP16 transactivator protein (Ace *et al.*, 1988). As discussed in Chapter 1, VP16, a

structural component of the virion, is the product of the essential γ gene (Vmw65 or UL48), that enters the host cell nucleus along with the viral DNA and transactivates the HSV1 IE genes (Batterson and Roizman, 1983; Campbell *et al.*, 1984). As an essential structural component of the viral capsid, it is not possible to outright delete this gene as this approach would produce a non-viable virus. Also, it would make propagation in culture impossible as VP16 is required for viral egress (Mossman *et al.*, 2000). Instead, Ace and colleagues (1989) inserted a 12bp fragment into the transactivating domain of the C-terminus of the protein. The disruption caused by the insertion of this fragment was sufficient to disrupt the domain of VP16 that interacts with the Oct-1 or HCF (O'Hare, 1993), while maintaining the ability of the protein to be incorporated into the virion during assembly. This virus was termed *in*1814 (Ace *et al.*, 1989). It should be emphasised that the insertion of the fragment does not abolish the transactivating function of VP16 but rather leads to a marked impairment (Mossman and Smiley, 1999) with a four to five fold reduction in the levels of ICP0 mRNA expressed in infected BHK cells (Ace *et al.*, 1989). Interestingly, the ICP4 RNA transcript levels remain unaltered (Ace *et al.*, 1989). In principle, the use of the *in*1814 mutation would complement the reduction in cytotoxicity achieved by deletions in IE genes. Indeed, it has been demonstrated in cell survival assays that it can reduce the deleterious effects of ICP4$^-$ mutants (Johnson *et al.*, 1994). The authors of this study proposed that the reason the *in*1814 mutation had such an effect in the cytotoxicity of the ICP4 deleted mutant was because the remaining transactivating activity of VP16 was insufficient to successfully initiate the production of transcripts from the ICP0, ICP22 and ICP27 promoters (Johnson *et al.*, 1994). Nearly a decade later, another research group produced a mutant with the whole of the VP16 transactivating domain deleted (Smiley and Duncan, 1997). This mutation, termed V422 (deleted from codon 422, C-terminal domain), displayed significantly reduced levels of the genes encoding ICP0 and ICP4 even in the presence of hexamethylene bisacetamide (HMBA), a synthetic IE gene transactivator (McFarlane *et al.*, 1992). In terms of the effects of this mutation on transcript levels, there is an apparent 20 fold reduction in the levels of ICP0 and ICP4 in Vero cells (Smiley and Duncan, 1997). A later study confirmed that a VP16 mutation combined with a reduction in ICP0 and ICP4

levels does indeed lead to lower levels of cytotoxicity to Vero cells (Preston *et al.*, 1997). The viruses used for these studies did not have any deletions in the IE genes. Instead a temperature-sensitive mutation in ICP0 was used. This mutation also utilised an ICP0 promoter that was less active that the wild type one. Culturing at limiting temperatures, these studies demonstrated that these vectors did not produce any IE genes and were thus able to persist in Vero cells following infection at a high MOI (5) (Preston *et al.*, 1997). Overall, the main dissimilarity between the two mutants is that the *in*1814 mutation has no effect on the levels of ICP4 transcripts while the V422 mutation results in a significant reduction. These differences could explain the cytotoxicity patterns observed with these vectors as emerged in comparative studies of ICP0/V422 and ICP0/*in*1814 double mutants where the ICP0/*in*1814 mutant appeared to be consistently more cytotoxic than the ICP0/V422 mutant even at low MOI (1) (Mossman and Smiley, 1999). The vector constructs used throughout this study carry the *in*1814 and not the V422 mutation. Since the cytotoxicity differences between these two mutants are likely to be due to the marked difference in the levels of ICP4, the inclusion of the *in*1814 mutation on a backbone that is already deleted for the presence of ICP4 would result in a vector that was no more cytotoxic than a vector carrying the V422 mutation (Lilley *et al.*, 2001b). The impaired ability of a VP16 inactivated virus in inducing IE gene expression has the added advantage that *in vivo*, the virus is forced to enter a latency-like state with low cytopathic effects, even though viral replication does occur at very high MOI (Ace *et al.*, 1989). This is an added advantage in terms of our aim of delivering genes to the CNS, as inducing latency would allow for our constructs to persist in the host cell and favour transgene delivery.

The deletion of essential IE genes such as ICP4 and ICP27 (DeLuca *et al.*, 1985; Sacks *et al.*, 1985), means that the virus is unable to enter a lytic life cycle. However in terms of delivering foreign genes to the injured CNS, more safeguards have to be put in place to ensure that the natural neurovirulence of HSV1 is abolished. This means that in addition to the deletion or inactivation of immediate early genes, it is necessary to disable the non-essential genes that come into play when establishing an infection in non-dividing cells such as

neurons. For example, ICP36 and ICP6 genes, encoding a thymidine kinase (tk) (MacLean *et al.*, 1991) and a ribonucleotide reductase respectively (Cameron *et al.*, 1988), as well as the γ34.5 gene, encoding the ICP34.5 neurovirulence factor (Chou *et al.*, 1990) are proteins that are mainly useful for the replication of HSV1 in neuronal cells. It therefore follows that preventing their expression could be beneficial. However, deletions in the above-mentioned genes alone are not equally efficient at reducing neurovirulence. Deletions in the tk gene result in some reduction in neurovirulence but only if the mutant virus is inoculated at a low titre (Palella *et al.*, 1988; Palella *et al.*, 1989). ICP34.5 deleted viruses have been shown to significantly impair the neurovirulent nature of HSV1. Deleting the ICP34.5 gene allows for a $1x10^6$ increase in LD_{50} compared to wild type HSV1 following direct inoculation in the brain of mice (Chou *et al.*, 1990). Even though, ICP34.5$^-$ (Coffin *et al.*, 1996) and ICP34.5/VP16 deletion mutants (McMenamin *et al.*, 1998) are avirulent when administered in either the peripheral or central nervous system, they are able to grow at near wild type titers *in vitro* using non-neuronal cell lines (Coffin *et al.*, 1996).

Other non-essential genes that have been studied for their contribution to cytotoxicity include: the IE genes ICP0, ICP22 and ICP47 and structural proteins of the virion such as Vhs (UL41 gene) (Coffin and Latchman, 1996). ICP0 deleted mutants are still able to grow in culture but at a much slower rate, especially when used at a low MOI (MOI=0.01) (Chen and Silverstein, 1992). In addition, *in vivo* experiments demonstrate that an ICP0 mutant largely retains the innate ability of HSV1 to be retrogradely transported to regions projecting to the inoculation site as opposed to an ICP4 deleted vector which did not produce a similarly widespread expression (Chiocca *et al.*, 1990). Thus, deleting the non-essential gene encoding ICP0, reduces the ability of the virus to replicate but, unlike the deletion in the ICP4 transactivator, it does not totally abolish this ability (Chiocca *et al.*, 1990). Another IE protein shown to be cytotoxic is ICP22 (Johnson *et al.*, 1994). The additional deletion of ICP22 in a virus already mutated for ICP4 and ICP27 can further reduce cytotoxicity as tested in neuronal and non neuronal cell lines (Wu *et al.*, 1996; Krisky *et al.*, 1998). As discussed in Chapter 1, the functions of Vhs include the non-specific degradation of host

mRNA and thus the inhibition of host protein synthesis (Read *et al.*, 1993). Even though such a function implies the creation of deleterious effects for the host, cells infected with an ICP4 and Vhs deleted or ICP4 and Vhs and VP16 mutant viruses do not appear to have any dramatic differences in cytotoxicity compared with ICP4 or ICP4 deleted and VP16-inactivated mutant viruses (Becker *et al.*, 1993). Since ICP4 (DeLuca *et al.*, 1985) and ICP27 (Sacks *et al.*, 1985) are essential viral genes, their removal renders the virus incapable of initiating a lytic cycle unless they are provided in *trans* by growing the mutant virus on a ICP4/ICP27 complementing cell line (DeLuca *et al.*, 1985; Chiocca *et al.*, 1990; Dobson *et al.*, 1990). Further consideration needs to be given to mutants also carrying the *in*1814 mutation. When the *in*1814 mutation has been combined with a deletion in an essential IE gene such as ICP4 then HMBA is inadequate at complementing the residual VP16 activity even if ICP4 is provided by the cell line *in trans* (Thomas *et al.*, 1999). In our laboratory, this problem was overcome following the creation of a cell line that can not only complement the deletions in ICP4 andICP27 genes but also complement the VP16 inactivating mutation as well. It is not possible to provide the HSV1 VP16 protein via a complementing cell line as this would allow the protein to be packaged into the new virions produced. This would cause the virus to be cytotoxic following a CNS injection, as the protein would lead to the transcription of IE genes.

Thomas *et al.* (1999) overcame this problem by constructing a cell line that expressed an analogue of the HSV1 VP16 protein that could not be packaged into the progeny virions but could still transactivate the IE gene promoters. This was the EHV1 gene 12 which encodes for the equivalent of VP16 in the equine HSV1 virus and had previously been shown to be able to transactivate HSV1 IE genes (Purewal *et al.*, 1994). Importantly, as there is very little DNA sequence homology between the two genes there is no risk of a homologous recombination of the EHV1-gene 12 into the VP16 locus in the disabled HSV1 genome. The resulting cell line was termed 27/12/M:4 and it was used for the production of high titre stocks of the highly disabled vectors used throughout this thesis (Thomas *et al.*, 1999).

3.1.2 Achieving long term transgene expression

As already mentioned in Chapter 1, in addition to combating cytotoxicity it is necessary to ensure that the vector can support the expression of the transgene for as long as possible following the successful transduction of the host cell. The long-term expression achieved with the expression cassettes incorporated in these vectors exploit the innate ability of HSV to remain transcriptionally active during latency by making use of specific elements from the LAT promoters (Palmer *et al.*, 2000; Lilley *et al.*, 2001b). The highly efficient expression cassettes employed here are the product of a substantial body of research from ours as well as other laboratories. The rationale that governed the production of the current cassettes is briefly reviewed in the paragraphs that follow (Lilley *et al.*, 2001a).

Early attempts at achieving transgene expression with disabled HSV1 viral vectors demonstrated that the location of insertion of the transgene in the viral genome as well as the choice of promoter, are key parameters that directly affect the expression pattern of the foreign gene. First attempts utilised viral promoters that were active during the lytic cycle of the virus. The preliminary expression cassettes that were constructed employed promoters such as ICP0, ICP4, ICP6, ICP8, gC and tk that were active during the lytic phase of the virus life cycle (Ho and Mocarski, 1988; Palella *et al.*, 1988; Chiocca *et al.*, 1990). These promoters were linked to the marker gene LacZ. These expression cassettes were all inserted via homologous recombination into the loci of genes also active during the lytic cycle. What all these early experiments had in common was that insertion of marker transgenes in place of viral genes under the control of promoters not normally active during latency did not result in long term expression of the transgene. Expression stops very quickly as soon as latency is established. For example, inserting lacZ into the ICP4 locus under the control of the ICP6 promoter, not active during latency, gives only short-term expression when delivered in the mouse CNS or PNS (Chiocca *et al.*, 1990). This was not necessarily a problem relating to the fact that the promoters used were of HSV1 origin as the same pattern emerged in other experiments where exogenous promoters such as CMV (Fink *et al.*, 1992) or the moloney murine leukaemia

virus (MMLV) long terminal repeat (LTR) (Lokensgard *et al.*, 1994) were placed into the US3 or gC loci respectively. Interestingly, when an MMLV LTR-lacZ construct was placed in an anti-sense orientation to ICP4 (thus inactivating it), expression in the mouse DRG continued for a period of 24 weeks following sciatic nerve inoculation (Dobson *et al.*, 1990), i.e.: long after the virus had entered latency. There must have been something relevant to the orientation and location of the insertion that allowed it to be active during latency, something that was not possible in any of the other locations this cassette had been inserted in previously. What these researchers did not know at the time was that by inserting the construct in an anti-sense orientation they placed the promoter in close proximity to the endogenous LAT region and in the same orientation as the LAT promoters. This impressive result therefore suggested that there might have been elements in the MMLV LTR promoter that together with the location of its insertion in relation to the latency promoters facilitated the long-term expression observed.

As already explained in Chapter 1 the production of LAT transcripts is under the control of two promoters LAP1 and LAP2. To recap, LAP1 is a TATA sequence promoter that is located 700 to 1300bp upstream from the of the actual LAT (8.3kb) (Batchelor and O'Hare, 1990; Zwaagstra *et al.*, 1990). LAP2 does not contain a TATA element and is located downstream of LAP1 and ~750bp upstream of the 2kb LAT intron (Goins *et al.*, 1994). These promoters have long been the target of attempts to exploit the fact that they remain active throughout latency. Initial studies into exploring their potential concentrated around the role of LAP1 as LAP2 was then unrecognised. What these initial experiments demonstrated was that in order for the LAP1 promoter to influence the longevity of transgene expression from an exogenous promoter, that promoter must be placed within the LAT region and at a specific distance from LAP1 itself. The evidence that led researchers to this realisation comes from several experiments. For example, when lacZ or NGF were inserted just downstream of the TATA element of the LAP1 promoter, transgenes expression was only supported for 21 days post inoculation (Margolis *et al.*, 1993). When the insertion site was placed ~800 bp further downstream from the TATA element from the LAP1 promoter

and within the endogenous LAT region then transgene expression could be supported for 56 days in mouse DRGs (Ho and Mocarski, 1989). In these experiments, the histological appearance of the resulting LacZ staining appeared unusual and punctate which the authors hypothesised may relate to physiological changes in the latently infected neuron relating to the transition from the acute to the latent stage of infection (Ho and Mocarski, 1989). Placing the LAP1 promoter at sites other than the endogenous LAT region abolishes any beneficial effect it may have in supporting long-term expression of transgenes. This was the case when Dobson and colleagues placed a LAP1-lacZ construct into the gC locus (Dobson *et al.*, 1995). No transgene expression was observed in this case. These findings further support the suggestion made by a number of different research groups that LAP1 on its own cannot support transgene expression in the long term.

The realisation that the region downstream of LAP1 (now known LAP2) was important for long-term expression, resulted from a number of additional experiments. When the *lacZ* gene was placed just downstream of the LAP2 either in the ectopic gC locus or in its native position in LAT, expression was maintained for more than 300 days in mouse trigeminal ganglia (Goins *et al.*, 1994). In reality however, the LacZ expression in this study was so weak that it could only be detected by PCR. In another study, the two promoters were combined. In this study, *lacZ* was fused with the encephalomyocarditis virus (EMCV) internal ribosome entry site (IRES), and inserted 1.5Kb downstream from the TATA box in LAP1 thus leaving LAP1 and LAP2 undisturbed. The authors reported that LacZ could be detected in cervical ganglia and cervical spinal cord sections at approximately three months and ten months respectively (Lachmann and Efstathiou, 1997). Again however, the transgene was present at very low levels and in only a handful of cells. The presence of LAP2 could also enable the LAP1 promoter to support the expression of foreign genes at HSV1 genome sites other than the endogenous LAT region. This was something that did not occur when LAP1 alone was placed at the gC locus (Dobson *et al.*, 1995). It was shown that it was possible for the LAP1 promoter to support the production of lacZ mRNA transcripts from the same gC locus but only when it

was placed upstream from the LAP2, and in the reverse orientation (Lokensgard *et al.*, 1997). In these experiments transgene transcript production could be detected in mouse DRGs at 28 days post footpad inoculation. This experiment demonstrated that in principle, the enhancer element in LAP2 could act at sites other than the endogenous LAT region. Lokensgard and colleagues (1997) conducted a series of experiments into the properties of LAP2. They concluded that LAP2 was *most* effective when it was placed downstream of LAP1 and in its natural orientation (Lokensgard *et al.*, 1997).

As mentioned previously the MMLV LTR promoter was unique in its ability to confer long term transgene expression during latency but only when placed in close proximity to the LAT region in the ICP4 locus (Dobson *et al.*, 1990; Lokensgard *et al.*, 1994). Further experiments were carried out aiming at finding out what was the mechanism behind this. In these experiments, the LAP1 promoter, without its TATA box, was fused with the MMLV LTR-*lacZ* (MMLV LTR-lacZ/LAP1^{TATA-}) cassette and inserted into the gC locus (Lokensgard *et al.*, 1994). Lokensgard *et al.* (1994) demonstrated that this chimeric promoter could support the expression of LacZ in murine DRGs for around 42 days post footpad inoculation. Therefore it appears that the MMLV LTR promoter can substitute for the LAP2 contribution in the long-term activity of LAP1. It has been suggested that the reason MMLV LTR and LAP2 appear to be capable of similar function may be a reflection of their ability to keep this region of DNA accessible to transcription factors (Lilley *et al.*, 2001a) such as the high mobility group (HMG) of proteins. These can directly bind and disrupt LAP2 (French *et al.*, 1996). Also, the local region has a dinucleotide content that is proportionally different to the rest of the HSV1 genome and could therefore reflect some structural characteristics that enable the LAP2 region to escape transcriptional silencing during latency (Coffin *et al.*, 1995).

This ability of LAP2 to maintain adjacent promoters in an active state would be useful in constructing vectors aimed at gene delivery to the injured CNS. This was exploited in our laboratory when cassettes were constructed carrying elements of the LAP2 and LAP1 regions that allowed exogenous promoters such

as CMV, RSV, NSE and MMLV LTR to sustain the expression of marker genes both in the PNS (Palmer *et al.*, 2000) and CNS (Lilley *et al.*, 2001b). In particular, in the expression cassettes constructed the exogenous promoter was placed at 700bp upstream of the 3' end of the LAP2 region and after a 1.4kb DNA fragment that has been named as LAT P2 in our laboratory (Palmer *et al.*, 2000). This location corresponds to 1.5kb downstream of the transcription initiation site for the primary LAT transcript. These expression cassettes were initially characterised following delivery to the CNS via sciatic nerve or footpad inoculations and were shown to be able to support the long term expression of transgenes (Palmer *et al.*, 2000). In later studies they were shown to be able to transduce spinal cord motor neurons retrogradely following a sciatic nerve inoculation (Perez *et al.*, 2004). These expression cassettes can be placed anywhere in the viral genome and not just into the LAT region. This is because they can act autonomously due to the presence of the LAT P2 region next to two exogenous promoters driving the expression of two (e.g. the pR20.5 expression cassette shown Figure 3.3.1-a, page 135) or one transgene (e.g. the pR19 expression cassette, Figure 3.3.2-a, page 138) (Palmer *et al.*, 2000). Indeed these vectors have been shown to support the expression of transgenes from ectopic sites of the viral genome for at least two months in murine DRGs following sciatic nerve inoculation (Palmer *et al.*, 2000).

3.1.3 Characteristics of the highly-disabled vectors used

The injured CNS environment is a very delicate tissue in terms of regeneration and places high demands on any tool designed for inducing regeneration. On the one hand the viral vector must be devoid of cytotoxicity while at the same time be able to support the long term expression of any potentially therapeutic transgene. Despite the difficulties posed, the current viral vector technology appears to combine both characteristics and can therefore be considered as a very powerful tool available for exploitation in terms of regeneration studies targeting the injured CNS. Deletions designed to reduce cytotoxicity, negatively impact the ability of the virus to support the long-term expression of transgenes. This is because when IE genes such as the ICP4 and VP16 transactivators are absent, the

viral genome is actively repressed very quickly following host infection (Preston and Nicholl, 1997). It has therefore been proposed that the task of developing a suitable viral vector system based on HSV1 is essentially a balance with each deletion in essential genes potentially resulting in a compromise in the efficiency of transgene delivery. Some researchers have even suggested that it might not be possible to produce a non toxic vector that is capable of sustaining a meaningful level of transgene expression (Samaniego *et al.*, 1998). A potential solution to this problem is to exploit the natural propensity of the virus to produce LATs by forcing the virus into latency.

As discussed in previous sections, effective reduction in cytotoxicity requires a combination of deletions in both essential and non-essential genes. Deletion of viral genes also enhances the capacity of HSV vectors to accommodate transgenes greater that 30Kb (Coffin and Latchman, 1996). The highly disabled HSV1 viral vectors utilised as the basis for the production of neurotrophin expressing vectors in this thesis, make use of the *in*1814 inactivating mutation and are deleted for ICP34.5, including ORF P. This double mutant is known by the term 1764. They also carry full deletions in the IE genes encoding ICP4 and ICP27 (Lilley *et al.*, 2001b). The initial characterisation of this backbone showed that it does not produce detectable levels of ICP0 on non-complementing cell lines, as shown by Western blotting (Lilley *et al.*, 2001b). The vectors used here are disabled to such an extent that they cannot replicate *in vivo* as they lack essential genes. Due to this they have to be supplemented in culture by especially designed cell lines in order to be propagated in high titre stocks (Thomas *et al.*, 1999; Lilley *et al.*, 2001b). Despite their multiple disabling deletions these viruses can support the expression of transgenes in transduced neuronal cells *in vivo* and *in vitro* in both the CNS (Lilley *et al.*, 2001b) and PNS (Palmer *et al.*, 2000). This is accomplished through the use of unique expression cassettes that make use of the LAT P2 region of the HSV1 genome, which is placed next to exogenous promoters. Out of a panel of expression cassettes produced in our laboratory, two were selected for the studies described in this thesis. Firstly, vector cassette pR20.5*vhs*, which allows the simultaneous delivery of two separate transgenes under the control of the RSV and CMV exogenous

promoters. One copy of this expression cassette was present for each copy of the viral genome as it was inserted into the locus of the *vhs* gene in the U_L region of the genome. The second cassette employed was the pR19CMV cassette, which can deliver a single gene under the control of the CMV promoter. This expression cassette however is inserted in the LAT region and is thus present twice per viral genome.

3.1.4 Rationale

The experiments employed in this chapter set out to examine whether there is a correlation between the location of the expression cassette employed in vector constructs and the pattern of transgene expression achieved following a single inoculation in the spinal cord. Specifically, it was hypothesised that placing the expression cassette in the endogenous LAT region would allow for more efficient transcription as the virus establishes latency. To test this hypothesis two vector constructs were compared for their ability to firstly achieve widespread transgene expression and secondly for their ability to support that expression in the long term. The first construct contained the ectopically placed pR20.5 cassette while the second construct contained the pR19 cassette, which is present in two copies within the endogenous LAT region.

It was also hypothesised that if expression in cortical pyramidal neurons is related to the level of deletion of viral endogenous genes, then by utilising a less disabled construct, such as HSV1.RL1$^+$/pR19CMV or HSV1.ICP27$^+$/RL1$^+$/pR19CMV, it would be possible to transduce cortical neurons. This could in turn translate in longer and more widespread transgene expression in cortical neurons compared to the more disabled HSV1.pR19CMV construct. The experiments that follow aim to measure differences between two vector constructs that bear different levels of disablement. The vectors were assessed their ability firstly to achieve long term expression and secondly for differences in their ability to target different neuronal populations following a single spinal cord inoculation. Based on the work presented here suitable HSV1 vectors were selected in order to construct new, neurotrophin expressing viral constructs that could potentially be used in various injured CNS paradigms.

3.2 Methods

The initial part of this study concentrated on the identification of optimally disabled HSV1 based vectors for use as a gene delivery tool for the CNS, in general and the spinal cord in particular. Vector mediated transduction of retinal ganglion neurons is examined in Chapter 6. As part of this study a series of different vectors were evaluated for their ability to efficiently transduce spinal cord neurons and support the expression of transgenes for a period of time thought sufficient to encourage regeneration, while at the same time having minimal cytotoxic effects.

3.2.1 Vectors utilised in Chapter 3

The following vectors were used in this study:

Virus	Abbreviation
1764/ICP27$^-$/P2$^-$/ICP4$^-$/pR20.5*vhs*	HSV1/P2$^-$.pR20.5*vhs*
1764/ICP27$^-$/ICP4$^-$/ pR19CMVLacZ	HSV1.pR19CMVLacZ
1764/ICP27$^-$/ICP4$^-$/RL1$^+$/pR19CMVLacZ	HSV1.RL1$^+$/pR19CMVLacZ
1764/ICP27$^+$/ICP4$^-$/RL1$^+$/pR19CMVLacZ	HSV1.ICP27$^+$/RL1$^+$/pR19CMVLacZ

Table 3.2.1-a: Vectors used in the vector backbone screening studies.

As discussed in the introduction to this Chapter the vector backbones that displayed the best practical compromise between vector toxicity and ability to efficiently transduce CNS neurons were those based on the 1764 backbone with the additional deletion of the ICP4 and ICP27 genes. This backbone, designated 1764/ICP27$^-$/ICP4$^-$, as well as the marker gene expressing vectors HSV1/P2$^-$.pR20.5*vhs* and HSV1.pR19CMV were all constructed by Dr C. Lilley in our laboratory and were available at the time when this study was initiated. Their construction has been previously published (Thomas *et al.*, 1999; Lilley *et al.*, 2001b) and is briefly summarised here. Vectors 1764/ICP27$^-$/ICP4$^-$/RL1$^+$/pR19CMVLacZ and 1764/ICP27$^+$/ICP4$^-$/RL1$^+$/pR19CMVLacZ were produced towards the end of this study by Dr J. Palmer in our laboratory and their construction is briefly reviewed here (unpublished work).

3.2.1.1 1764/ICP27⁻/ICP4⁻ & 1764/ICP27⁻/P2⁻/ICP4⁻ (HSV1) backbones

All viruses were derived from the HSV1 strain 17*syn*$^+$. The term 1764 is used to designate a vector that contains the *in*1814 mutation in the VP16 gene and has the gene encoding ICP34.5 and ORF P completely deleted between HSV1 *nt*124,945 to *nt*125,723 (GenBank file **HE1CG**). The term ICP27⁻ refers to the deletion of nucleotides HSV1 *nt*113,273 to *nt*116,869 (*Mlu*I - *Mlu*I). This means that the backbone also has genes UL54, UL55 and UL56 completely deleted. UL54 encodes the IE protein ICP27 which is essential while UL55 and UL56 are both non-essential genes (Howard *et al.*, 1998; Lilley *et al.*, 2001b). This backbone was designated 1764/ICP27⁻/ICP4⁻ (HSV1) and is often referred to as "fully disabled" for the purposes of this thesis. In addition, the 1764/ICP27⁻/P2⁻ /ICP4⁻ designated backbone (HSV1/P2⁻) was deleted for the endogenous LAT P2 region [HSV1 *nt*118,768 (*Dde*I) – *nt*120,470 (*Hpa*I)] in order to avoid any recombination events with the pR20.5 expression cassettes that also contains a LATP2 element (Palmer *et al.*, 2000).

3.2.1.2 Vector 1764/ICP27⁻/P2⁻/ICP4⁻/pR20.5vhs (HSV1/P2⁻.pR20.5vhs)

The pR20.5vhs expression cassette was available at the time of initiation of this study and details of its construction have already been published (Palmer *et al.*, 2000; Lilley *et al.*, 2001b). Briefly, the pR20.5 cassette contains a central LATP2 region (HSV *nt*118866 to *nt*120219; GenBank file HE1CG) flanked by two promoters in a back-to-back orientation. Firstly, the CMV promoter (Invitrogen) driving the expression of eGFP (Clontech) and secondly the RSV promoter (pRcRSV-Invitrogen) driving the expression of lacZ (HindIII-BamHI; Pharmacia). The pR20.5 expression cassette was flanked by vhs gene sequences [HSV1 *nt*90313 (*Kpn*I) - *nt*91854 (*Nru*I) and *nt*91854 (*Nru*I)- *nt*93660 (*Hpa*I)] and this construct was termed pR20.5vhs. Vector 1764/ICP27⁻/P2⁻/ICP4⁻ /pR20.5vhs (designated HSV1.pR20.5vhs for the purpose of this thesis and depicted in Figure 3.3.1-a, page 135) was constructed by homologous recombination of the pR20.5vhs cassette into the UL41 gene in the HSV1 genome. The cassette was recombined into the unique *Nru*I site (HSV1 *nt*91854) resulting in the insertional inactivation of the UL41 gene (Thomas *et al.*, 1999; Palmer *et al.*, 2000; Lilley *et al.*, 2001b).

3.2.1.3 Vector 1764/ICP27⁻/ICP4⁻/pR19CMV (HSV1.pR19CMV)

The construction of the pR19CMV cassette has been previously described (Palmer *et al.*, 2000). Briefly, lacZ was cloned from pCH110 (HindIII-BamHI; Pharmacia) and the full length CMV promoter from pcDNA3 (654bp; Invitrogen). In the GFP versions of the pR19 cassette the gene was the eGFP (Clontech) version. The cassette was cloned into the HSV1 LAT region using a 3.5kb *Not*I fragment (HSV1 *nt*118,443 to 122,029) inserted into pGem5 plasmid (Promega). The pR19CMV cassette, with either GFP or lacZ, was cloned between the *Bst*XI sites (HSV1 *nt*120,219 to *nt*120,413). For the construction of the recombinant vectors the pR19CMV cassette, expressing either LacZ or eGFP, was recombined into the two endogenous LAT regions between the two *Bst*XI sites (HSV1 *nt*120,220 - *nt*120,408) of the fully disabled backbone (1764/ICP27⁻/ICP4⁻; Figure 3.3.2-a, page 138). The pR19CMV cassette was inserted in both endogenous LAT regions and therefore exists in two copies in each recombinant genome.

3.2.1.4 Vectors 1764/ICP27⁻/ICP4⁻/RL1⁺ & 1764/ICP27⁺/ICP4⁻/RL1⁺

As discussed in section 3.2.1, both these vectors were produced by Dr J. Palmer and their details have not yet been published. Both ICP34.5 and ICP27 genes were sequentially reinserted into their original locations into the viral genome. Both genes were inserted via homologous recombination with appropriate plasmid constructs.

3.2.2 Cell culture and high titre viral stock production

Production of high titre viral stocks, suitable for *in vivo* inoculation was carried out as outlined in the methods section and was based on the use of the 27/12/M:4 cell line. This was a BHK-derived cell line designed to complement the high level of deletions in essential genes in the 1764/ICP27⁻/ICP4⁻ deletions (Thomas *et al.*, 1999). The 27/12/M:4 cell line is specifically designed to propagate vectors that carry mutations in the vmw65 and ICP4 genes. Firstly, it encodes a non-HSV1 homologue of the vmw65 gene derived from the EHV1 gene 12. Secondly, complementation for the ICP4 deletion is achieved via the inclusion of

plasmid pMAMZeo/ICP4 that allows for the expression if ICP4 [HSV1 *nt*127,167-*nt*131,187 (*Mse*I - *Bst*EII)] under the control of the MMLV promoter. The complementing capacity of this cell line was maintained by ensuring that passaged cells were maintained in 800μg/ml of neomycin (G418) and 750 μg/ml of phleomycin D1 (Zeocin; Cayla, Toulouse, France). 3mM HMBA was included in the growth media of viruses as they contained the *in*1814 mutation. Details of stock production, concentration and purification are detailed in Chapter 2.

3.2.3 *In vivo* transgene delivery via disabled viral vectors

The experiments described in this Chapter aim to explore the ability of the variably disabled vectors described in section 3.2.1 (page 126) to achieve maximal transgene delivery to the CNS and the spinal cord in particular. Even though there are subtle technical differences between them, the experiments described here were carried out primarily in uninjured animals with the exception of vector HSV1.pR19CMV that formed the basis of future, neurotrophin expressing vector construction and is therefore examined for its ability to deliver genes to the acutely and chronically injured spinal cord (sections 3.3.2, page 137 and 3.3.3 page 151).

3.2.3.1 Spinal cord lesions prior to vector delivery

All experiments were carried out on deeply anaesthetised adult Lewis rats (≈200g). Before proceeding further, all animals were given a subcutaneous dose of the analgesic Finadyne (2.2 mg/kg, Schering-Plough). A longitudinal incision was made along the spine at either the cervical or lumbar level. This was followed by a deeper incision, separating the superficial muscle layers and further dissecting the deeper muscle layers by blunt dissection. The vertebrae were exposed and a bilateral laminectomy was performed using malleus scissors. The dura matter was then cut with micro-scissors. In cases where no injury was required, the procedure continued as outlined in the following section (3.2.3.2, page 130), and the virus under consideration was delivered. Alternatively, a lesion of the corticospinal tract (CST) was performed prior to virus delivery.

Using irridectomy scissors, an incision severing both the ascending dorsal columns and the dorsal corticospinal tracts and extending to the central canal was performed. The dura and overlying muscle tissue was repaired using silk sutures prior to closing the skin with Michel clips.

3.2.3.2 Spinal cord Microinjection of disabled HSV1 vectors

Injections were carried out as described in the general methods Chapter (Chapter 2, section 2.7.3). Briefly, a 25µl Hamilton syringe fitted with a fine needle (outer Ø 300 µm) was used to administer the vector at a 1.5 mm depth into the uninjured spinal cord or within the lesion site in CST lesioned animals. Viral suspensions were injected at a rate of 0.5 µl/min using a micro-pump. The spinal cord lesions described in this Chapter vary depending on which vector was being compared and their deletions. Therefore, each of the results sections briefly outlines the nature of the experiment conducted and the relevant lesion that was performed. In the spinal cord injection experiments described here the majority of animals had not received a lesion prior to being inoculated with the vector under consideration. In section 3.3.3, the transgene delivery achieved with vector HSV1.pR19CMVLacZ was evaluated in the setting of a CST lesion which was performed either immediately prior or one month following the lesion.

3.2.3.3 Vector microinjection into the cerebellum of adult rats

Injections were carried out as described in the general methods Chapter (Chapter 2) and in the previous section. Briefly, a 25µl syringe fitted with a fine needle (outer Ø 300µm) was used to administer the vector at a 4 mm depth into the cerebellum. Again, viral suspensions were injected at a rate of 0.5µl/min using a micro-pump. The coordinates used for the injection were: Rostral-Caudal: 11.5mm using bregma as zero, Medial-Lateral: 1.5mm, Dorsal-Ventral: 3 - 5mm.

3.2.4 Detection of viral mediated marker gene transduction

Vector mediated neuronal transduction following an *in vivo* delivery was assessed based on the expression of either LacZ or GFP. Detection of the marker genes was carried out as outlined in the methods section (Chapter 2). Briefly,

GFP was visualized under fluorescence as soon as tissue was sectioned while LacZ expression was based on the detection of β-gal detection. Sections were incubated at 37°C with the 0.1% solution of X-gal in a solution containing 5mM potassium ferrocyanide, 5mM potassium ferricyanide and 2mM $MgCl_2$. It is worth pointing out that vector HSV1.pR20.5vhs is capable of expressing both LacZ and GFP.

3.2.5 Mapping of LacZ gene expression in virally transduced neurons

Both spinal cords and brains from injected animals were extracted three weeks post inoculation. Spinal cords were sectioned transversely at 40μm thickness while brains were again cut into coronal sections of 100μm thickness. After they were reacted for the presence of LacZ, as outlined in the previous section, all sections were mounted onto slides maintaining their correct sequence. Each section was then inspected for the presence of LacZ positive neuronal somata. All sections, spinal cord or brain that contained LacZ positive neuronal somata were drawn onto tracing paper using a camera Lucida attached to a microscope. Each of the neuronal groups were then identified using a reference atlas (Paxinos, 1994). These results are collectively presented in section 3.3.3, page 151.

3.2.6 GFAP and OX42 Immunostaining of spinal cord sections

As shown in section 3.3.2 (page 137) 40 μm thick spinal cord sections, previously reacted for the presence of LacZ, were subsequently immunolabelled for GFAP and OX42. For GFAP staining a monoclonal, anti-GFAP (clone GA5) antibody was used at a 1:600 dilution (Sigma Chemical Company, Ltd, Poole, Dorset, UK). Similarly, for identifying activated microglia a mouse anti-rat OX42 (clone CD11) antibody was used at a dilution of 1:200 (Sera-Tech Biologicals Inc.). The preparation of spinal cord sections, as well as the steps taken for each of the above immunolabelling procedures is described in the methods section (Chapter 2). One type of negative control was employed in each of the immuno-histochemistry techniques described in this Chapter. For either GFAP or OX42 staining, three LacZ-stained sections for each procedure were

isolated and processed as normal but the primary antibody incubation step was omitted. On processing, neither of the secondary antibodies at the dilutions detailed above led to any non-specific staining (results not shown).

3.2.7 Histological characteristics of CNS resident cells

The discrimination between neuronal and non-neuronal cell populations in this thesis was based mainly on morphological appearance. Findings were referenced against well-documented histological features (Young and Heath, 2003).

NEURONS display great morphological variation depending on which part of the nervous system they reside in. Despite this, all cells share the same basic structure with a large soma (30-50μm wide), a relatively large and eccentrically placed nucleus and a prominent nucleolus. Neuron somas give rise to a slender, straight axon and characteristic highly branched dendritic trees.

ASTROCYTES have small round to oval cell bodies that are <10μm wide with evenly dispersed, pale chromatin and they are present in both grey and white matter. They have a star-shaped appearance, imparted by the multipolar, branching cytoplasmic processes that emanate from the cell body containing the characteristic cytoplasmic protein, GFAP. Overall, they have a pale cytoplasm that is rich in Golgi complexes and GFAP. The GFAP filaments are either aggregated in fascicles (*protoplasmic*) or diffusely dispersed throughout the cytoplasm (*fibrous* astrocytes).

OLIGODENDROCYTES have a denser, more homogeneous chromatin, found within a small, characteristically round, lymphocyte-like nucleus. They are small cells (≈8μm wide) and their cytoplasm contains a large number of mitochondria, microtubules and lysosomes. They vary in morphology from large euchromatic nuclei and pale cytoplasm to heterochromatic nuclei and dense cytoplasm. In sections where oligodendrocytes have been transduced by a LacZ expressing vector, these cells can be distinguished by their nuclei and their processes which wrap around numerous adjacent axons.

MICROGLIA are small cells (5-8μm wide) with very little cytoplasm, which forms fine, short and highly branched processes. They have an elongated, irregular shaped nucleus with clumped chromatin. In response to injury, microglia lose their ramifications and transform into large amoeboid phagocytes (Figure 1.3.2-c) (Stence *et al.*, 2001).

3.3 Results

3.3.1 Characterization of the fully disabled HSV1/P2⁻.pR20.5*vhs*

The only available construct thought to be suitable for gene delivery to the injured spinal cord at the time when this study was initiated was vector 1764/ICP27⁻/P2⁻ICP4⁻/pR20.5*vhs* (HSV1/P2⁻.pR20.5*vhs*). As depicted in Figure 3.3.1-a, this construct is based on the use of the pR20.5 expression cassette that allows for the simultaneous expression of two separate transgenes (Palmer *et al.*, 2000). In theory, this would make monitoring of transgene expression *in vivo* easier compared with other options such as "tagging" the neurotrophin. Additionally, as discussed in the introduction, the fully disabled backbone seemed suitable for delivery to the spinal cord in particular due to its minimal cytotoxic effects to cultured DRG or hippocampal neurons (Palmer *et al.*, 2000; Lilley *et al.*, 2001b). The ability and efficiency of HSV1/P2⁻.pR20.5*vhs* to transduce spinal cord neurons to express both LacZ and GFP in adult rats was evaluated. Adult rat spinal cords (n=6) were inoculated with $2.5x10^4$ pfu of HSV1/P2⁻.pR20.5*vhs* at the L4-L5 level. Transduced cells were identified based on the criteria listed in section 3.2.7.

As it can be seen in Figure 3.3.1-b (adjacent sections shown) infected cells can express both GFP (**Ai**) and LacZ (**Aii**) transgenes at the one-week time point. Interestingly, this vector does not appear to preferentially transduce neurons despite the innate neurotropism of HSV1. As shown in the enlarged panels in Figure 3.3.1-b, HSV1/P2⁻.pR20.5*vhs* can transduce neurons (**Bi & ii**) as well as glial cells (**Biii**) around the injection site. This is not surprising since HSV1 is innately able to infect glial cells as they too express heparan sulphate proteoglycan which allows viral particle attachment (Vahlne *et al.*, 1980; Marsh *et al.*, 2000). Even though microscopically there appeared to be both astrocytes and oligodendrocytes transduced these were not differentiated with cell specific markers such as GFAP or Myelin Oligodendrocyte Specific Protein (MOSP). Some neuronal cells display a distinct peri-nuclear and punctate LacZ staining pattern (Figure 3.3.1-b, panel **Bii**, ↓) indicative of low level LacZ expression or localisation in defined cytoplasmic structures (Ho and Mocarski, 1989).

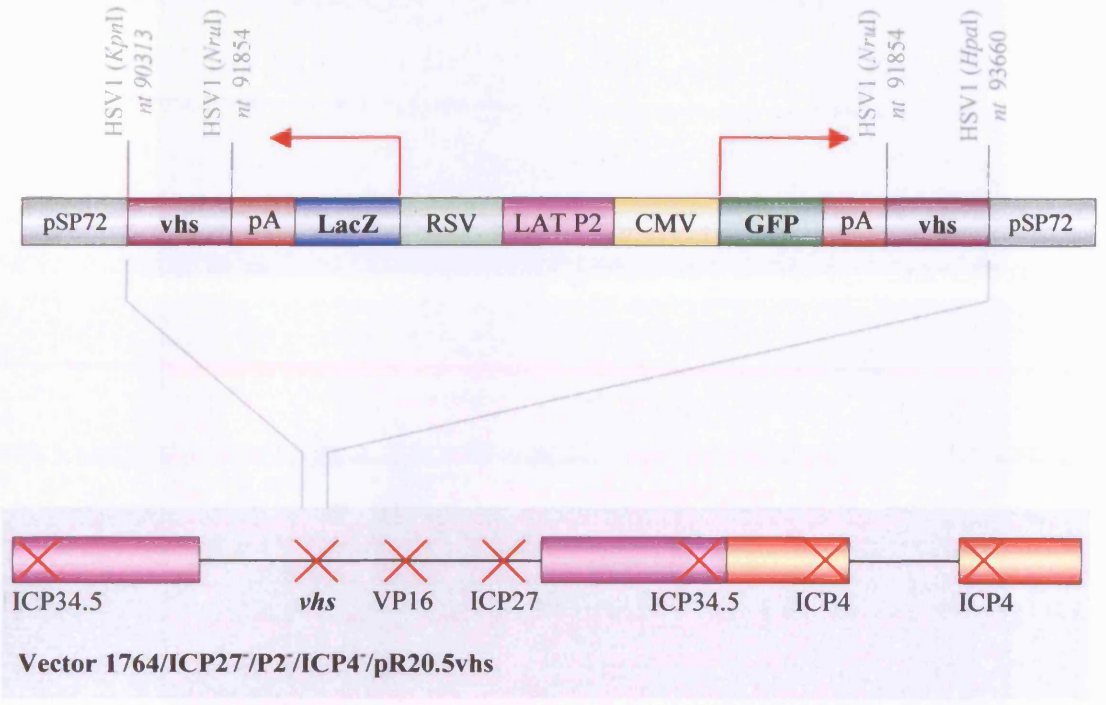


Figure 3.3.1-a: Schematic representation of the HSV1/P2$^-$.pR20.5*vhs* viral backbone.

Two heterologous promoters, CMV and RSV, flank the central LAT P2 element. The promoters are arranged in a back-to-back orientation, designed to allow the simultaneous expression of two exogenous genes such as the two marker genes encoding GFP and LacZ (Palmer *et al.*, 2000).

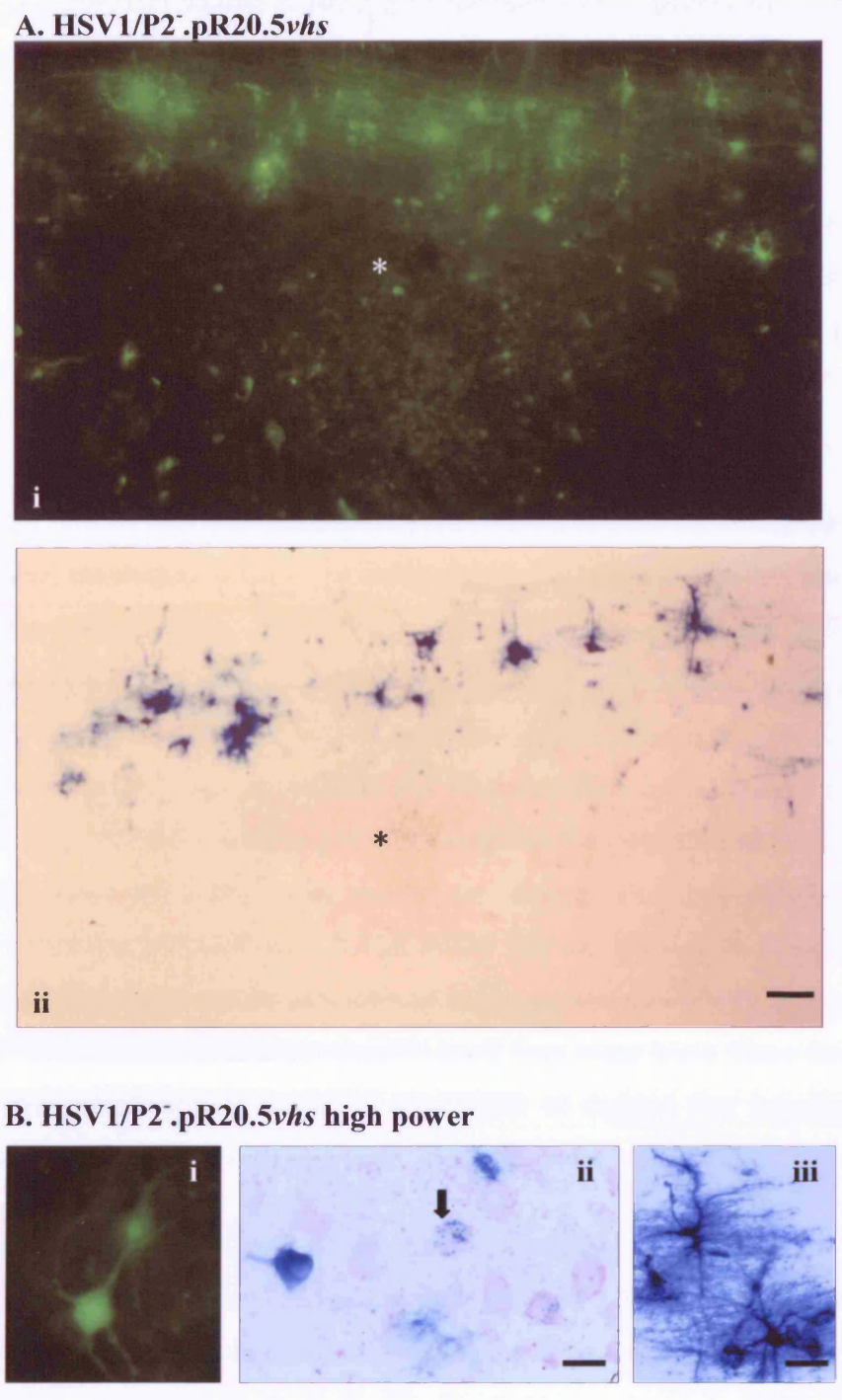


Figure 3.3.1-b: HSV1/P2⁻.pR20.5*vhs* mediated expression of GFP and LacZ in spinal cord.

Micrographs of spinal cord sagittal sections, showing GFP (**Ai, Bi**) and LacZ (**Aii, Bii** and **iii**) expressing transduced cells at the one-week time point, located close to the injection site of HSV1/P2-.pR20.5vhs ($2.5x10^4$ pfu). Higher magnification of cells in other sections reveals that there was transduction of both neurons (**Bi, ii**) and glia (**Biii**). Arrow indicates punctate staining in spinal cord neurons (Injection site *).

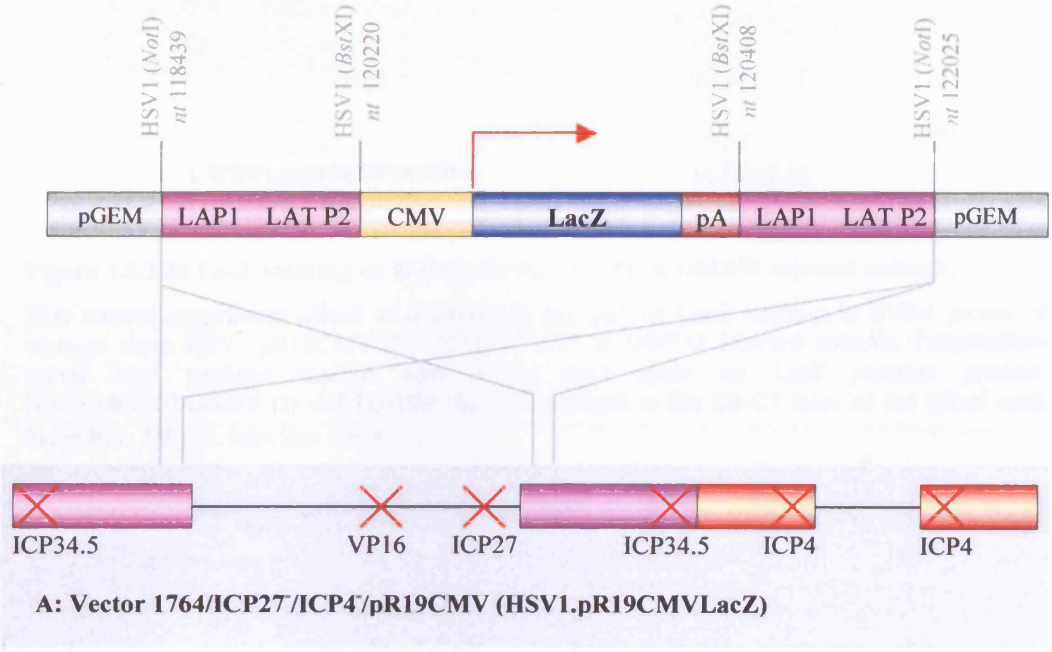
Scale bars: **Aii**=50μm, applies to **Ai**; **Bii**=40μm, applies to **Bi**, **Biii**=15μm.

3.3.2 Characterisation of the highly disabled HSV1.pR19CMV vector

Unlike HSV1/P2⁻.pR20.5*vhs*, HSV1.pR19CMV (1764/ICP27⁻/ICP4⁻ /pR19CMVLacZ) contains two copies of the pR19CMV cassette with a single transgene under the control of the CMV promoter. Each pR19CMV expression cassette is inserted into each of the two endogenous regions of the viral genome (Figure 3.3.2-a, page 138). The properties of HSV1.pR19CMVLacZ in terms of transgene delivery were examined following a single injection of $2.5x10^5$ pfu at the C6-C7 level of the adult rat spinal cord (n=6). At the three-week time point, transgene expression in the spinal cord was evaluated.

Prior to proceeding, it is important to point out that, as Figure 3.3.2-b (page 139) demonstrates, incubating spinal cord sections with X-Gal stain does not result in non-specific LacZ staining. This is true when animals have been injected with $2.5x10^4$ pfu of vector HSV1.pR19CMVGFP (n=1) (Figure 3.3.2-b, panel **i**), or DMEM (n=1) (Figure 3.3.2-b, panel **ii**), which is used in viral stock formulation. The only area that develops some pale blue discolouration is the choroid plexus but that is very rare and occurs mostly following direct inoculation into the brain parenchyma (personal observation, results not shown). However, this is only observed following incubations in X-Gal buffer that are allowed to proceed for longer than 8 hours and can be exacerbated in the presence of 5% CO_2. It could be argued that since the injection process in itself does cause some tissue damage in the animals examined, it would be reasonable to suggest that injuring the neural tissue does not lead to any non-specific LacZ staining.

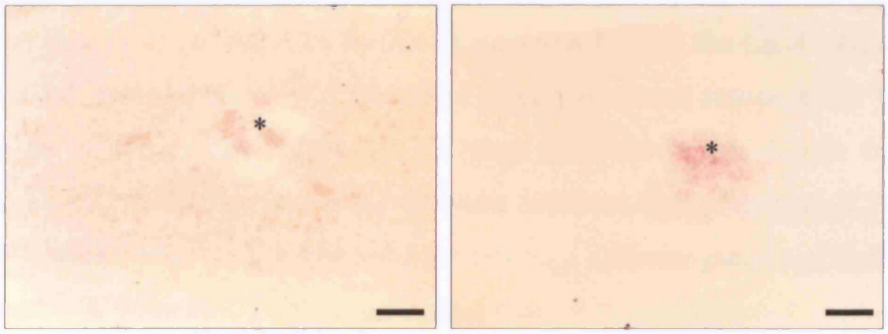
Preliminary experiments using a GFP expressing construct of the HSV1.pR19CMV backbone demonstrate that a single $2.5x10^4$ pfu inoculation (n=2) is sufficient to maintain GFP expression for at least one month following inoculation (Figure 3.3.2-c, panel **i**). On the contrary, a single $2.5x10^4$ pfu inoculation with HSV1/P2⁻.pR20.5*vhs* vector (n=2) does not lead to any transduction at the same time point (Figure 3.3.2-c, panel **ii**). The fact that GFP expression is under the control of the same promoter between the two vectors demonstrates that the differences in expression patterns at the one-month time point are due to differences between the two backbones rather than the expression cassettes themselves.



A: Vector 1764/ICP27⁻/ICP4⁻/pR19CMV (HSV1.pR19CMVLacZ)

Figure 3.3.2-a: Schematic representation of HSV1.pR19CMV based viral backbones.

The HSV1.pR19CMVLacZ vector incorporates two copies of the pR19CMV cassette inserted in both of the two endogenous LATP2 regions.

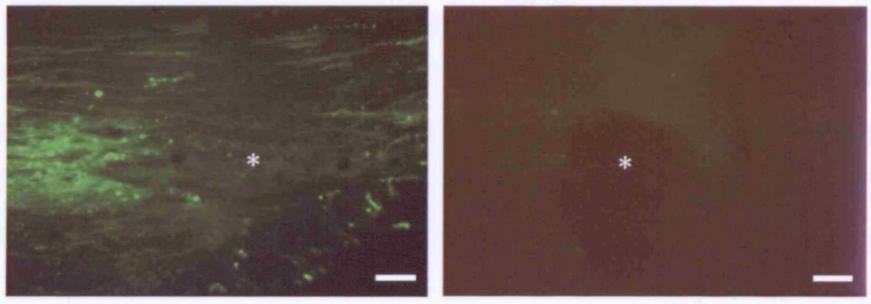


i. HSV1.pR19CMVGFP (stained for LacZ)

ii. DMEM

Figure 3.3.2-b: LacZ staining on HSV1.pR19CMVGFP & DMEM injected animals.

This control experiment aimed to demonstrate the lack of LacZ staining in X-Gal processed sections from HSV1.pR19CMVGFP ($2.5x10^4$ pfu) & DMEM injected animals. Longitudinal spinal cord sections reacted with X-Gal stain show no LacZ reaction product. HSV1.pR19CMVGFP (**i**) and DMEM (**ii**) were injected at the C6-C7 level of the spinal cord. Scale Bar: 250μm, injection site *.



i. HSV1.pR19CMVGFP

ii. HSV1/P2$^-$.pR20.5*vhs*

Figure 3.3.2-c: HSV1.pR19CMVGFP & HSV1/P2$^-$.pR20.5*vhs* expression, 1-month p.i.

Sagittal sections of C6-C7 spinal cord previously injected with either vector HSV1.pR19CMVGFP (**i**: $2.5x10^4$ pfu, n=2) or vector HSV1/P2$^-$.pR20.5*vhs* (**ii**: $2.5x10^4$ pfu, n=2) demonstrating that only HSV1.pR19CMVGFP transduced cells express the GFP marker gene at one-month post injection. Bright axonal processes and cell somata are present (**i**). Scale Bar: 250μm, injection site *.

It was therefore decided that assessment of the expression patterns achieved with either of these vectors could be further characterised using the LacZ rather than GFP marker gene as this would provide a more permanent record at the longer time point of one month. Figure 3.3.2-b (page 139) shows that despite the fact that HSV1.pR19CMV vector carries the same deletions in the IE genes ICP4 and ICP27 as vector HSV1/P2$^-$.pR20.5*vhs*, it displays a different pattern of transgene expression. A three cm, longitudinal spinal cord section (displayed in panel **A**) clearly demonstrates that this vector retains the innate ability of HSV1 to be retrogradely transported from the injection site, along axonal processes to reach the neuronal somata. The neurons transduced are not only spinal cord neurons within the injection site (panel **D**) but also sensory neurons in ipsilateral DRGs (panel **C**) as well as neurons located at the medulla, pons and midbrain level that also project in the vicinity of the inoculum (panel **B**). The exact nature of these neurons is examined in section 3.3.3 (page 151). HSV1.pR19CMVLacZ appeared to confer significantly better expression than that observed with vector HSV1/P2$^-$.pR20.5*vhs*. It was therefore decided to examine the transgene expression achieved with each of these two vectors in parallel. Again the same parameters were used and each vector was delivered at the C6-C7 level of adult rats via a single $2.5x10^4$ pfu inoculation. It is important to clarify here that the number of virus particles utilised for the comparison of the two vectors was adjusted so that an equal amount of virus was delivered in each group of animals. This was necessary, as the HSV1/P2$^-$.pR20.5*vhsv* vector could not be grown at as high a titre as the HSV1.pR19CMVLacZ vector. Neuronal transduction was evaluated at 3 days, 2 weeks and 1 month (n=4 per vector, per time point). Figure 3.3.2-e (page 143) demonstrates that the two vectors vary in their ability to support transgene expression at one-month post inoculation. The compilation in Figure 3.3.2-e represents the best examples of each batch of animals, per time point. Even though there were some differences between animals in each group, these were not extensive enough to suggest that there were big variations in the amount of virus delivered via the current protocol. Vector HSV1.pR19CMVLacZ (panels **B**) appear to not only transduce more spinal cord neurons around the lesion site but also to support the expression of LacZ for at

Figure 3.3.2-d: HSV1.pR19CMVLacZmediated neuron transduction in the spinal cord.

A. Very low magnification micrograph of a longitudinal (80μm) section through the C6-C7 level spinal cord of a rat that had received an injection of HSV1.pR19CMVLacZ ($2.5x10^5$ pfu). LacZ expression is strong in both somata and axons (dark blue/black) in both grey matter (red) and white matter (pale central region) with evidence of retrograde transport to neurons projecting to the injection site (*). The inset shows a serial section of the injection site and the adjacent region, which is revealed at higher magnification in panel **D** where neurons and axons display strong LacZ transduction. **B.** Transduced neurons in the pons, medulla and midbrain levels below the dark red granule cell layer of the cerebellum of the same rat. **C.** Strongly transduced neurons and axons (↓) within the ipsilateral DRG project to the injection site

Scale bars: **A**=400μm, inset 300μm; **B**=1mm; **C**=150μm; **D**=100μm.

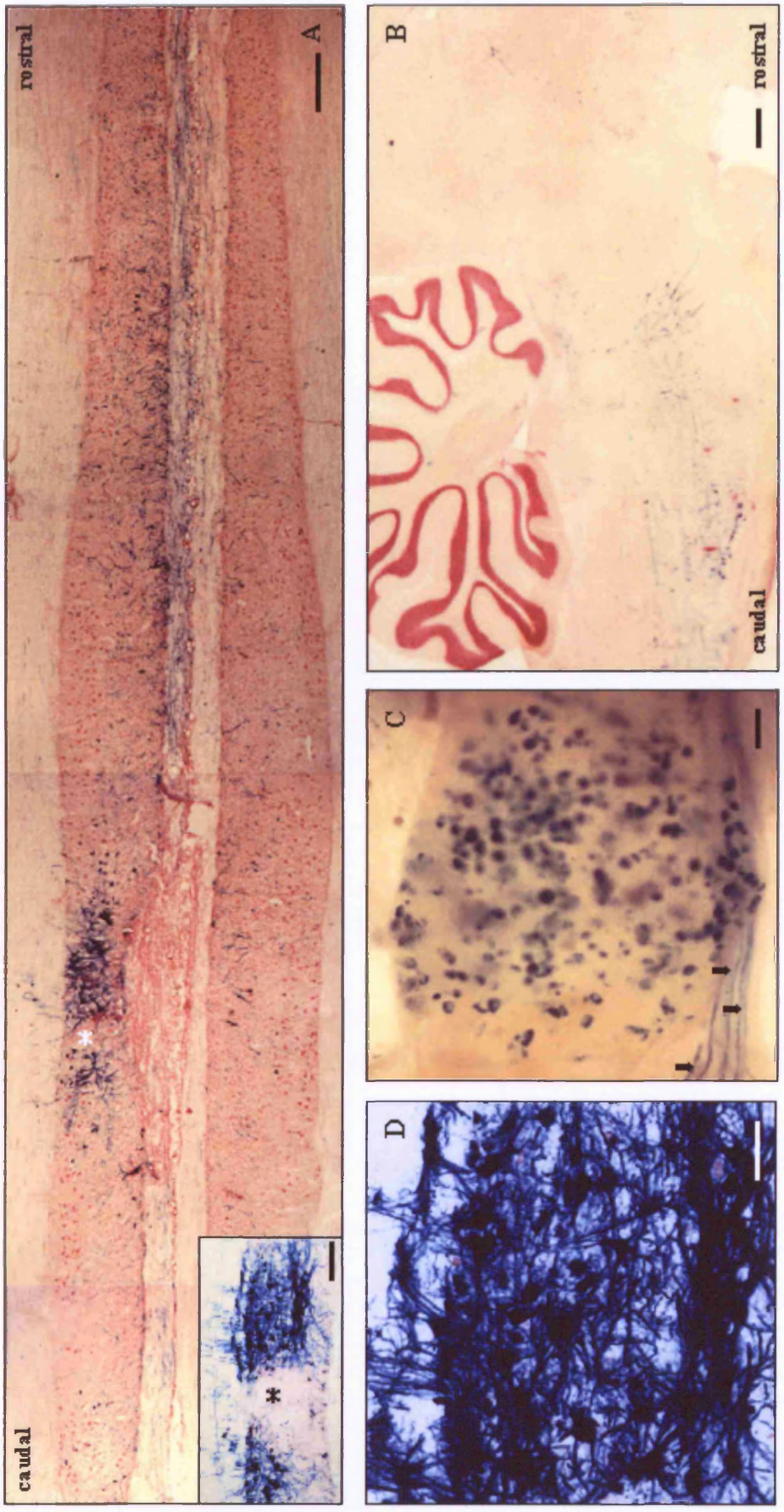
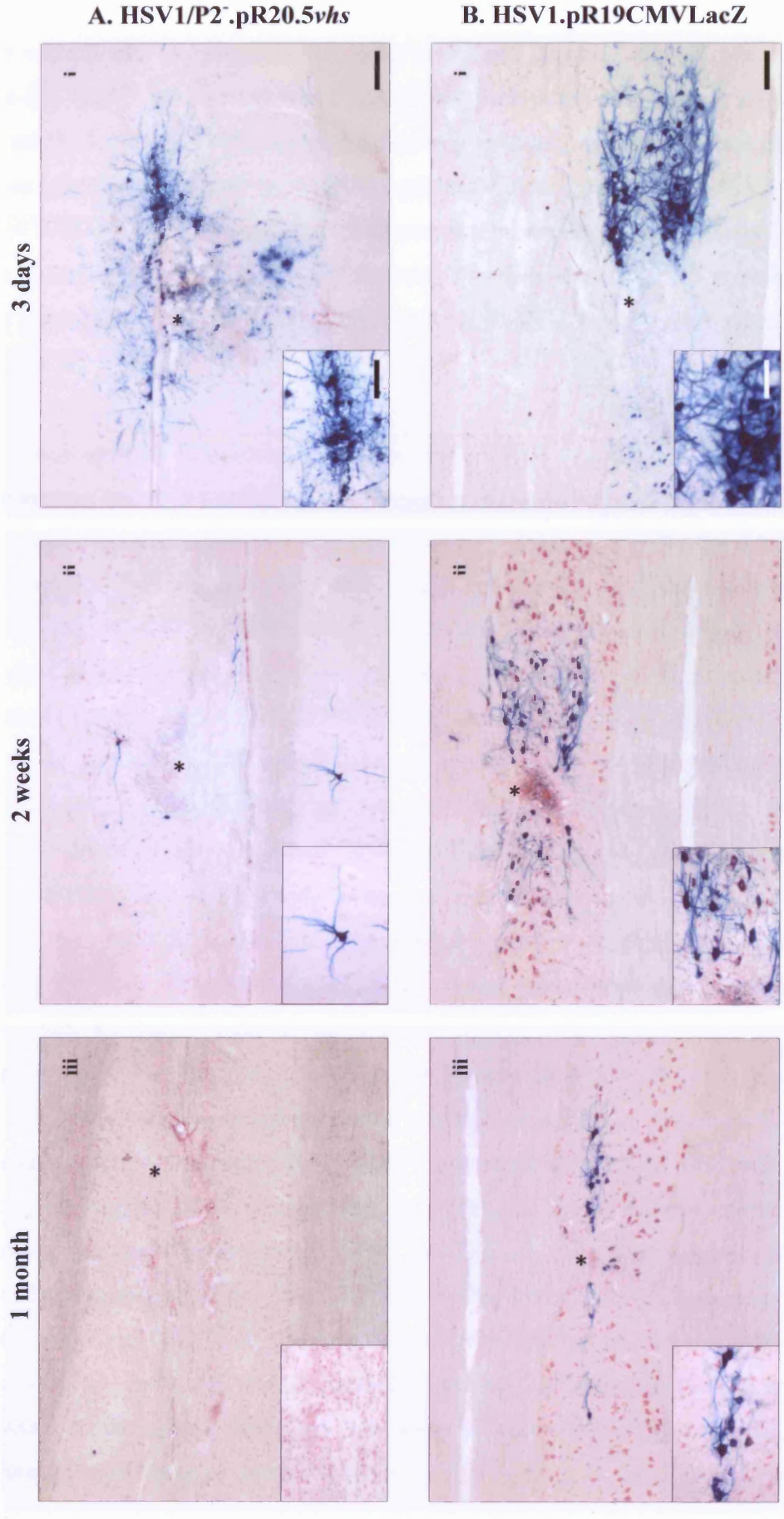

rostral
caudal
A
B
rostral
caudal
C
D

Figure 3.3.2-e: HSV1/P2-.pR20.5/vhs versus HSV1.pR19CMVLacZ transduction.

HSV1/P2$^-$.pR20.5*vhs* ($2.5x10^4$ pfu) **(A)** or HSV1.pR19CMVLacZ ($2.5x10^4$ pfu) **(B)** were inoculated at the C6-7 level. LacZ expression was examined on longitudinal (40μm) sections at 3 days **(i)**, 2 weeks **(ii)** and 1 month **(iii)** later. LacZ expression achieved with HSV1.pR19CMVLacZ is more widespread, stronger at the one month post-administration and selective to neurons compared with HSV1/P2$^-$.pR20.5*vhs*, which mediated weaker transduction in neurons and glia. The insert panels in each photograph depict examples of transduced neurons at higher magnification (Injection site *).

Scale bars: **Ai**=250μm, applies to **Aii** & **Aiii**; **Bi**=250μm, applies to **Bii** & **Biii**; all insets, 200μm.


A. HSV1/P2⁻.pR20.5vhs
B. HSV1.pR19CMVLacZ
3 days
2 weeks
1 month
i
ii
iii

least one-month, the longest time point examined (panel **Biii**). Between the three-day (panel **Bi**) and two-week (panel **Bii**) time points there was a gradual and subtle decline in LacZ expression that was continued in the later time point of one month. In contrast to the HSV1.pR19CMVLacZ vector, the HSV1/P2$^-$.pR20.5*vhs* construct was not able to support the expression of LacZ in the long term. LacZ levels declined sharply between the three-day (**Ai**) and two-week time point (panel **Aii**) but was absent by the end of the one-month time point (panel **Aiii**).

The initial promise that vector HSV1.pR19CMVLacZ demonstrated as a CNS gene therapy tool was reinforced when its performance was tested in animals that had previously received a dorsal column lesion in L4-L5 (n=4, Figure 3.3.2-f, panel **Aii**, †). Two weeks following the lesion, all animals were inoculated with $2.5x10^5$ pfu of HSV1.pR19CMVLacZ vector and the expression of LacZ was examined three weeks later. A whole mounted spinal cord (panel **Ai**) emphasises the strong levels of transgene expression surrounding the injection site (panel **Ai**, insert). In addition, LacZ stained neuronal processes can be seen leaving the spinal cord and entering either the dorsal or ventral root (panel **Ai**, ▼). The extent to which DRG neurons were transduced varies depending on the location of the injection site. For example in another animal, transduced DRG neurons can be seen next to a very strongly stained ventral root (both ipsilateral), indicating a very efficient transduction of ventral spinal cord motor neurons (panel **Aiii**, ▼). This is easier to see in a coronal section from the same animal. Here, ventral spinal cord motor neurons can be seen next to their axons (panel **Aii**, ▼). Unfortunately, despite repeated attempts at trying to transduce CST neuronal somata by modifying the delivery parameters to the spinal cord, such as altering the injection depth or delivering the same viral load in multiple aliquots, it proved an unattainable target. This inability to transduce neurons was somewhat unexpected at the time. However further investigations from projects carried out in parallel to this study did throw some light into the reasons behind this expression feature (further discussed in section 3.4). Importantly, the pre-lesioning of the spinal cord does not seem to affect the retrograde virally mediated transduction of brain stem neurons projecting to the injection site

(panel **Aiv**). Looking closely at the tissue surrounding the HSV1.pR19CMVLacZ injection site (Figure 3.3.2-d, panel **D**, page 141 & Figure 3.3.2-e, panel **Bi**, page 143), there appears to be a distinct lack of transduced glial cells. This is in direct contrast to the pattern observed with the HSV1/P2⁻.pR20.5*vhs* vector (Figure 3.3.1-b, page 136, panel **Biii** & Figure 3.3.2-e, panel **Ai**, page 143). It is possible that the HSV1.pR19CMVLacZ vector also displays a predilection for glial cells. However this may be difficult to detect due to the strong LacZ staining seen within cells around the injection site.

It was also decided to investigate whether the expression of high level of LacZ would cause inflammation in the *in vivo* CNS environment. In order to take a closer view at the transduced neurons, non-lesioned animals were injected with $2.5x10^5$ pfu of HSV1.pR19CMVLacZ vector (n=4) and 21 days later were stained with LacZ and examined for the presence of astrocytes and microglia at the lesion site. Coronal sections from the same HSV1.pR19CMV transduced animal shown in Figure 3.3.2-f, was stained with GFAP for astrocytes (panel **Bi-iii**) and OX42 for the presence of microglia (panel **Biv-vi**). At low magnification it is evident that the motor neurons of the left hand side ventral horn are strongly expressing the transgene (panels **Bi**). Note that the transduction pattern was unlike that seen in the lesioned animal depicted in the coronal section in panel **Aii**, which displays bilateral ventral horn staining. This is because in the case of the animal in panel **Aii** the injection took place closer to the midline thus reaching bilateral groups of ventral horn motor neurons. The animal sections shown in panel **B** however received the injection deep into the left hand side ventral horn. The injection site, which can be seen at low (panels **Bi** & **iv**) and high magnification (panels **Bii, iii, v** & **vi**), has caused a significant local disruption (panel **Bii** & **v**) but its cause is thought to be mechanical and attributed to the flexible needle used to carry out the stereotactic injection, further discussed in section 3.4 (page 166).

Close inspection of the GFAP staining pattern demonstrates that even adjacently to a cluster of LacZ expressing neurons, there is a lack of transduced astrocytes

(panel **Biii**). None of the GFAP stained astrocytes were found to be expressing LacZ (panel **Biii**, ↓). Admittedly, the pattern observed in this particular section may not be a true representation of the whole of the transduced spinal cord. However, it is fairly obvious that this vector displays a strong neuronal preference.

In terms of the astrocyte morphology, there appeared to be a somewhat stronger labelling at the edges of the immediate injection site where most of the tissue damage has occurred (panel **Bii**). These astrocytic processes appeared to be denser and more elongated while their cytoplasm is more strongly stained for GFAP compared to areas distal to the area of mechanical damage. Thick astrocytic processes were present in the area of maximum tissue damage (panel **Bii**, ←) amongst the LacZ stained axons of ventral motor neurons seen within the injection site (panel **Bii**, ▼). The astrocytes surrounding the virally transduced neuronal somata appear to be less densely stained for the presence of GFAP, with finer processes and a paler cytoplasm (panel **Biii**, $\downarrow^{1, 2, 3}$). Overall, there does not seem to be gross evidence of reactive astrogliosis around the neuronal somata that would be indicative of viral or transgene toxicity.

In addition to the LacZ and GFAP staining, the same sections were stained for the presence of the microglia/macrophages using the OX42 marker (Figure 3.3.2-f, panels **Biv-vi**). It should be emphasised here that OX42 does not distinguish between microglia and infiltrating macrophages and although the intensity of OX42 staining is greater on activating microglia, resting cells are also OX42-positive. Nevertheless, OX42 staining was maximal at the site of injection where tissue damage was severe (panel **Biv**) and appears to correlate with the boundaries of the necrotic region. Given the fact that there is such a strong LacZ expression in neurons of the left ventral horn it was somewhat surprising not to see a high level of OX42 immunofluorescence around the neuronal somata due to the levels of the β gal protein produced. It should be acknowledged that this crude technique does not rule out the possibility that there is at least some peri-neuronal inflammation that could be seen using a more

sensitive detection method. It is however plausible to suggest that with such high levels of transduction, both in terms of the number of neurons transduced but also in terms of the levels of the foreign gene expressed from transduced neurons, there was no significant microglia/macrophage presence around the neurons themselves. This indicates that there is little cytotoxicity with this vector.

Perhaps it would have been more appropriate to examine more closely the morphology of microglia in the grey matter and especially the areas surrounding the transduced neurons for evidence of activation such as loss of ramification or expression of activation specific markers such as ED1. There is, however, a high number of microglia/macrophages in the area of the injection site. It is most likely that these are infiltrating macrophages responding to the mechanical damage (panel **Bv** & **vi**). On higher magnification (panel **Bvi**) the majority of the cells within the injection site displayed a rounded morphology typical of infiltrating macrophages (panel **Bvi**, $\downarrow^{\alpha}$).

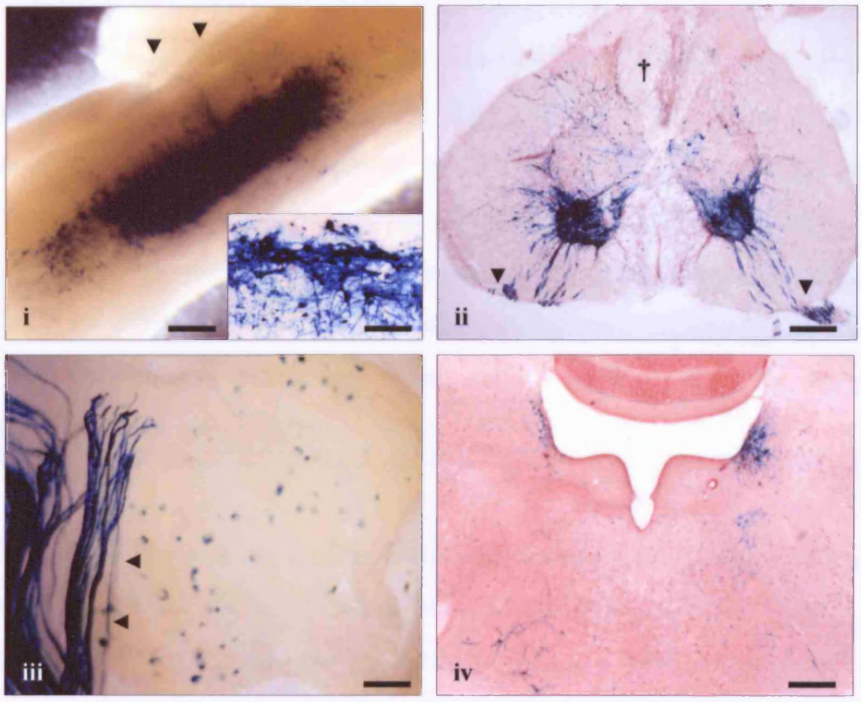
Figure 3.3.2-f: OX42 & GFAP staining of HSV1.pR19CMVLacZ transduced SC neurons.

A. Vector HSV1.pR19CMVLacZ ($2.5x10^4$ pfu) was injected at the L4-L5 level of a pre-lesioned spinal cord. LacZ expression in transduced neurons is strong as it is observed in a whole mounted spinal cord (**Ai** inset shows higher magnification of the transduced spinal cord region). Ventral, spinal cord motor neurons are transduced and can express the transgene for at least 21 days (**Aii**). DRG (**Aii**) and brainstem (**Aiv**) neurons projecting to the lesion site are also retrogradely transduced.

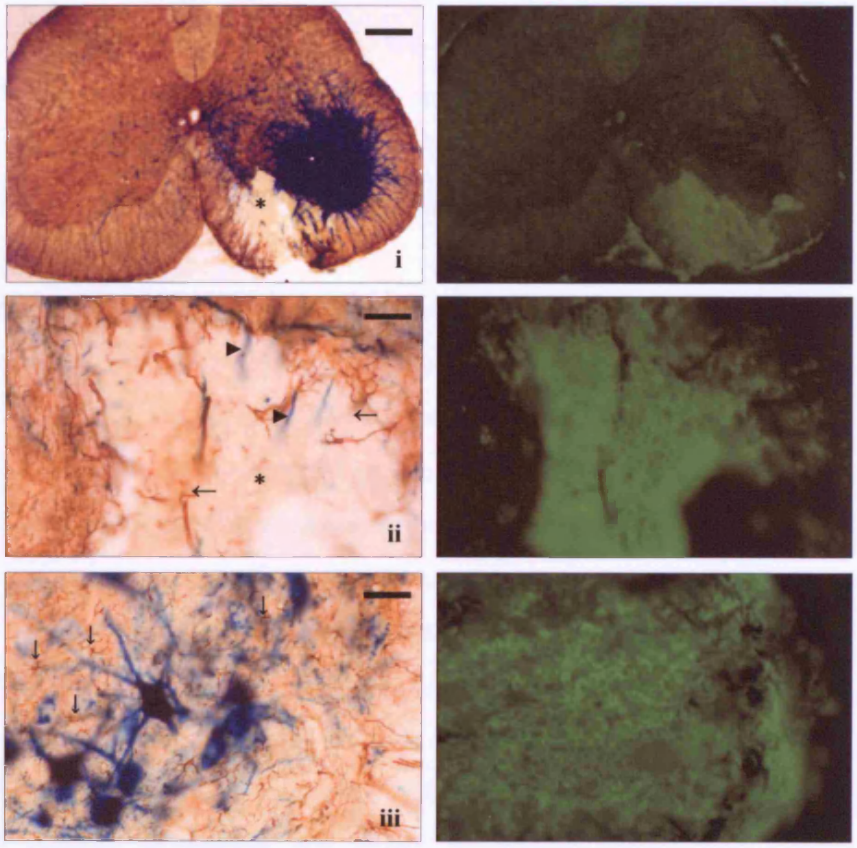
B. The same spinal cord section was processed sequentially for LacZ, GFAP (light brown, **Bi-iii**) and OX42 (green, **Biv-vi**). Around the LacZ expressing neurons (dark blue) the GFAP positive astrocytes (**Biii**) appear to have a normal morphology while there is a lack of OX42 upregulation (**Bvi**). OX42 (**Biv-vi**) immunoreactivity is however enhanced at the site of tissue destruction and is suggestive of the presence of activated microglia or infiltrating macrophages (Axonal processes ▼, astrocytes ↓, infiltrating macrophages $\downarrow^{\alpha}$, Injection site *, lesion site †).

Scale bars: **Ai**=800μm, inset=250μm; **Aii**=350μm; **Aiii**=200μm; **Aiv**=400μm; **Bi**=800μm, applies to **iv**; **Bii**=50μm, applies to **v**; **Biii**=50μm, applies to **vi**.

A. HSV1.pR19CMVLacZ mediated transduction in the injured SC



B. Effect of HSV1.pR19CMVLacZ transduction on OX42 & GFAP levels



3.3.3 Examination of HSV1.pR19CMV mediated transduction

As shown in the previous sections the HSV1.pR19CMV vector retains the innate ability of HSV1 to be retrogradely transported from axonal terminals to the corresponding neuronal somata. Prior to initiating any regeneration experiments utilising this vector it was thought necessary to explore exactly what types of neuronal populations could be transduced following a single injection in the spinal cord at the lumbar level. This series of experiments presented the opportunity to also explore whether the virus behaved differently in terms of which populations are transduced when it was injected into an acutely or chronically lesioned spinal cord.

Animals were split into three groups. Each group was assigned three animals and each animal within each group received a single injection of $2.5x10^5$ pfu of HSV1.pR19CMV vector into the L4-5 level. Group A (n=4) only received the vector inoculation. Groups B and C (n=4 each) first received a dorsal column hemisection lesion at the L4-5 level. Group B was injected with the same amount of vector two weeks following this lesion while Group C received the vector immediately after the lesion was performed. All animals were sacrificed three weeks following vector inoculation and the expression of the transgene was mapped from spinal cord through to the brain.

The figures in the following pages are compiled by selecting representative drawings from the animal that demonstrated the best transduction in each of the treatment groups. On the whole, vector HSV1.pR19CMVLacZ transduces a variety of different neuronal types in nuclei including: ventral and dorsal horn, reticular formation, vestibular nucleus, locus coeruleus, inferior olivary nucleus, inferior colliculus, trigeminal nuclei, Edinger-Westphal and hypothalamic nuclei. Overall, there were no obvious differences between the types of neurons that were transduced by the HSV1.pR19CMVLacZ vector in the non-injured (Figure 3.3.3-a), acutely injured (Figure 3.3.3-b) and chronically injured lumbar spinal cord setting (Figure 3.3.3-c). It must be emphasised that this study was designed with the aim of evaluating any major qualitative rather than quantitative differences in transduced neuronal populations. In order to confirm that the

nature of the neuronal groups transduced in non-lesioned (Group A) and chronically lesioned (Group B) spinal cords were similar, one animal from each group was selected (Rat A and Rat B respectively). Each of the two selected animals demonstrated the strongest and most widespread LacZ transduction within its group. Approximately 180 sections per animal were analysed and neuronal cell numbers for each neuronal group were counted. The collated data for Rat A (representative rat in Group A) and Rat B (representative rat in Group B) were tabulated.

Tract	Rat A	Rat B
Nucleus Tractus Solitarius	2	3
Vestibular Nucleus	11	54
Reticular Formation	82	580
Raphae Nucleus	99	62
Inferior Olivary Nucleus	26	3
Central Gray	36	17
Locus Coeruleus	186	364
Edinger-Westphal	31	5
Trigeminal Nucleus	30	3
Inferior Colliculus	1	10
Medial Geniculate Nucleus	2	5
Ventro-basal Thalamic Nuclei	1	2
Hypothalamic Nuclei	32	4

Table 3.3.3-a: HSV1.pR19CMVLacZ mediated transduction of neuronal groups
Number of transduced neuronal cell bodies for each representative rat from the non-lesioned (Group A) or injected with vector 2 weeks after a dorsal column hemisection (Group B). Only LacZ expressing neuronal somata were counted while LacZ positive axons were not included in this table. Note the similar distribution of transduced neurons in disparate brain locations e.g. nucleus tractus solitarius in caudal brainstem to hypothalamic nuclei more rostrally.

As it can be seen in Table 3.3.3-a, there are some differences in the numbers of transduced neurons even though the same classes of neurons were transduced in either of the two representative animals. However, it is more plausible that the variation in the numbers noted could reflect normal variations in the method of delivering the inoculum rather than variations in the efficiency of transduction between the two groups.

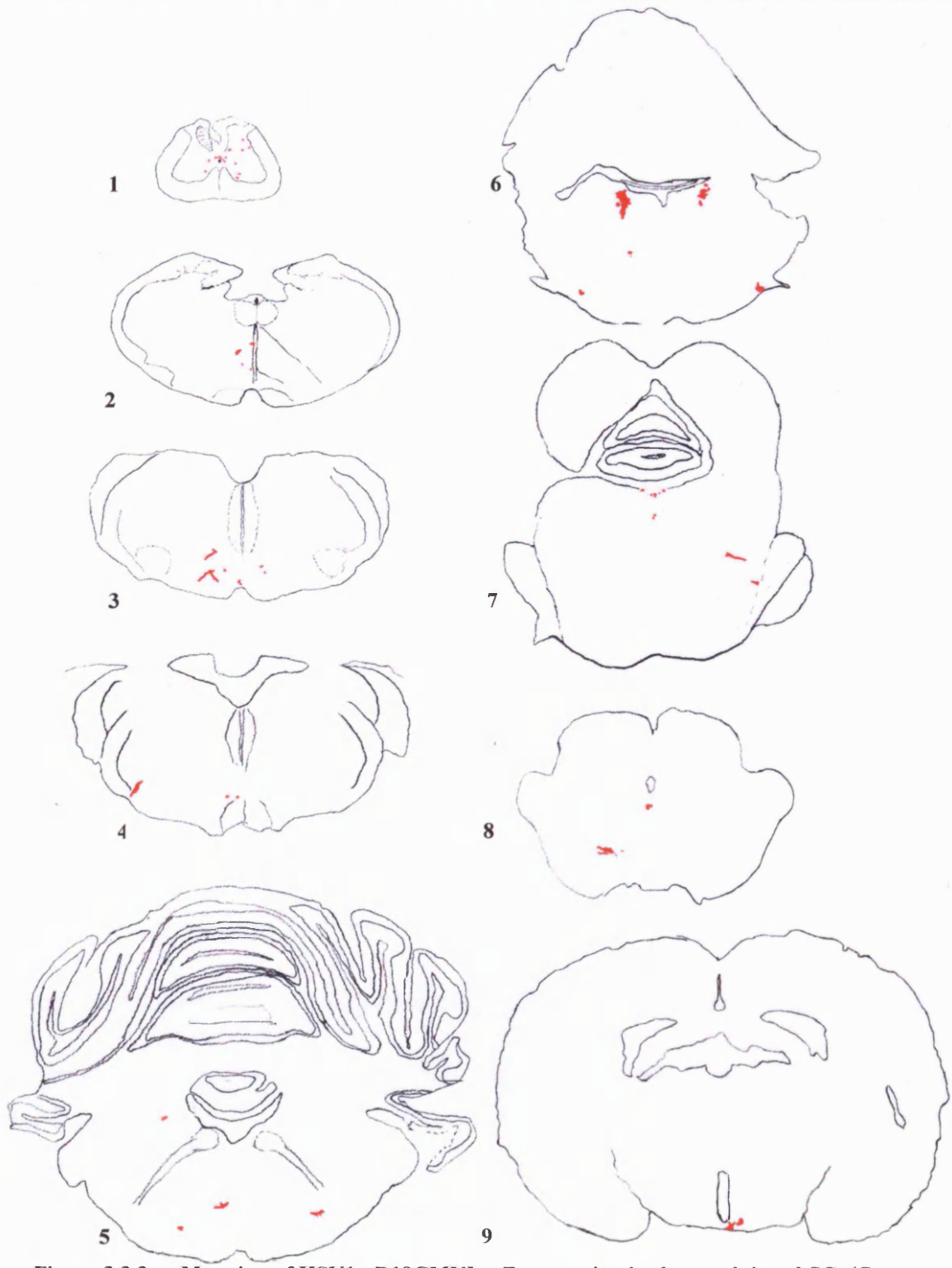


Figure 3.3.3-a: Mapping of HSV1.pR19CMVLacZ expression in the non-injured SC. (Group A) Neurons transduced via a single injection in the lumbar spinal cord of adult, non-pre-lesioned, lumbar spinal cord. Transduced neurons (shown in red) include: **1:** Ventral and dorsal spinal cord neurons, **2:** Vestibular nucleus, raphae nucleus and reticular formation neurons, **3:** Reticular formation and olivary nucleus neurons, **4:** Trigeminal nucleus and reticular formation nuclei, **5:** Vestibular and reticular formation nuclei, **6:** Locus coeruleus, reticular formation and olivary nucleus neurons, **7:** Raphae and trigeminal nuclei, **8:** Oculomotor and red nucleus neurons, **9:** Hypothalamic nuclei. Many of these transduced neurons are shown in photomicrographs on the adjacent page. *Note* the similarity of location of the retrogradely transduced neurons to that in Group B, chronically injured SC Figure 3.3.3-b and in Group C, acutely injured SC Figure 3.3.3-c. Scale bars: Spinal cord=500μm; Locus coeruleus=500μm, inset=150μm; Raphé=450μm; Reticular formation=500μm, inset=150μm; Red nucleus=700μm, inset=150μm.

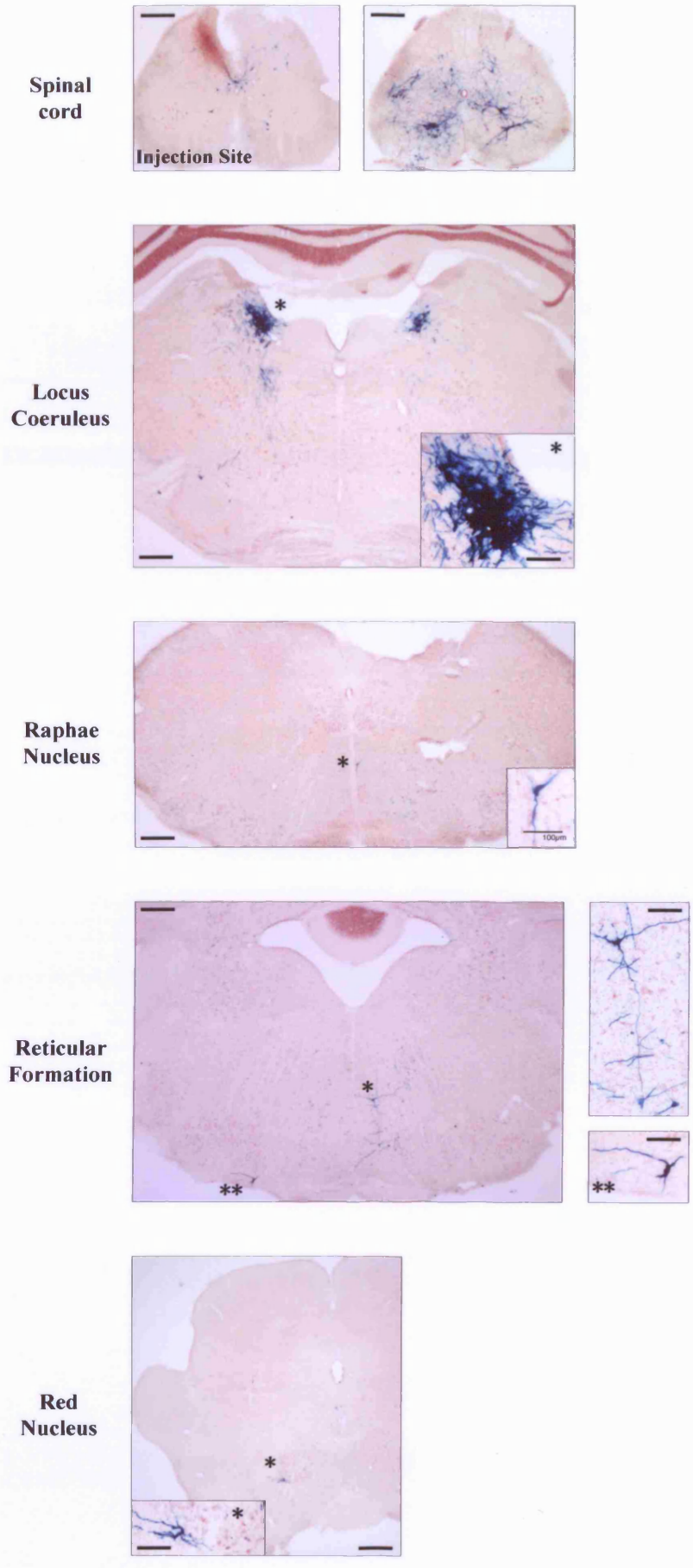
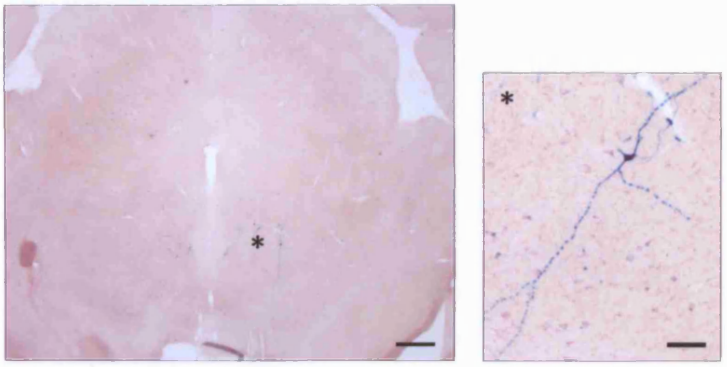

Spinal cord
Injection Site
Locus Coeruleus
Raphae Nucleus
Reticular Formation
Red Nucleus

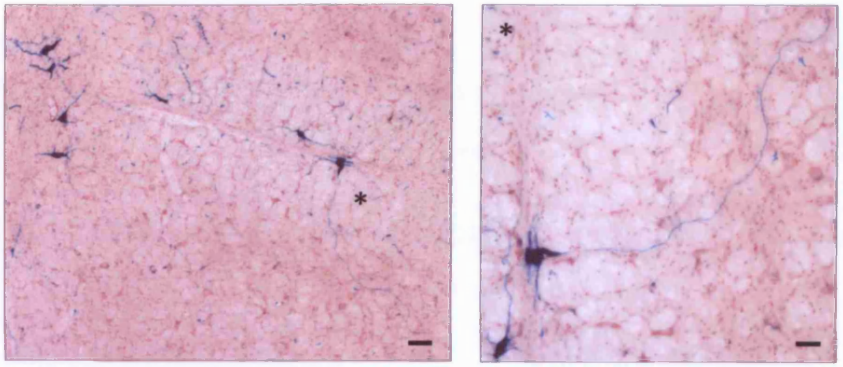
Figure 3.3.3-b: Mapping of HSV1.pR19CMVLacZ expression in the chronically injured SC.

(Group B) Neurons transduced via a single injection in the chronically lesioned CST in the lumber spinal cord. Transduced neurons (shown in red) include: **1:** Ventral and dorsal horn neurons, **2:** Reticular formation neurons, **3:** Reticular formation and vestibular nucleus neurons, **4:** Locus coeruleus, reticular formation and olivary nucleus neurons **5:** Inferior colliculus, locus coeruleus, trigeminal and reticular formation nuclei, **6:** Edinger-Westphal and central grey neurons, **7:** Hypothalamic nuclei. Many of these transduced neurons are shown in photomicrographs on the adjacent page. Note the similarity of location of the retrogradely transduced neurons to that in Group A, non-injured SC (Figure 3.3.3-a) and in Group C, acutely injured SC (Figure 3.3.3-c). Scale bars: Reticular formation: low power=500μm, high power=100μm; Raphé: low power=200μm, high power=100μm; Olivary nucleus: low power=200μm, high power=50μm; Oculomotor nucleus: low power=500μm, inset=50μm.

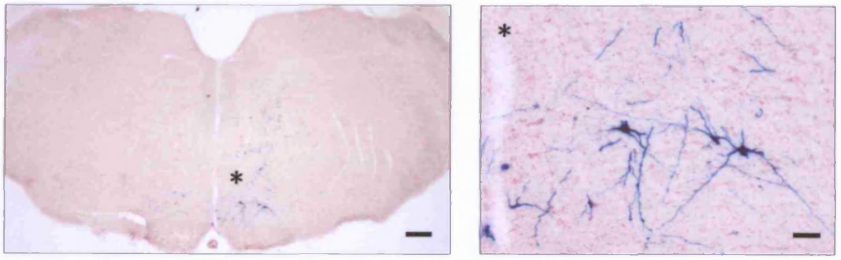
Reticular Formation



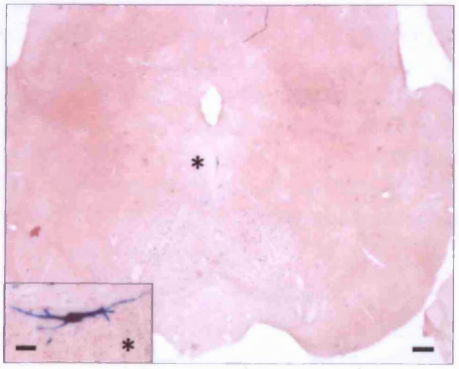
Raphae Nucleus



Olivary Nucleus



Occulomotor Nucleus



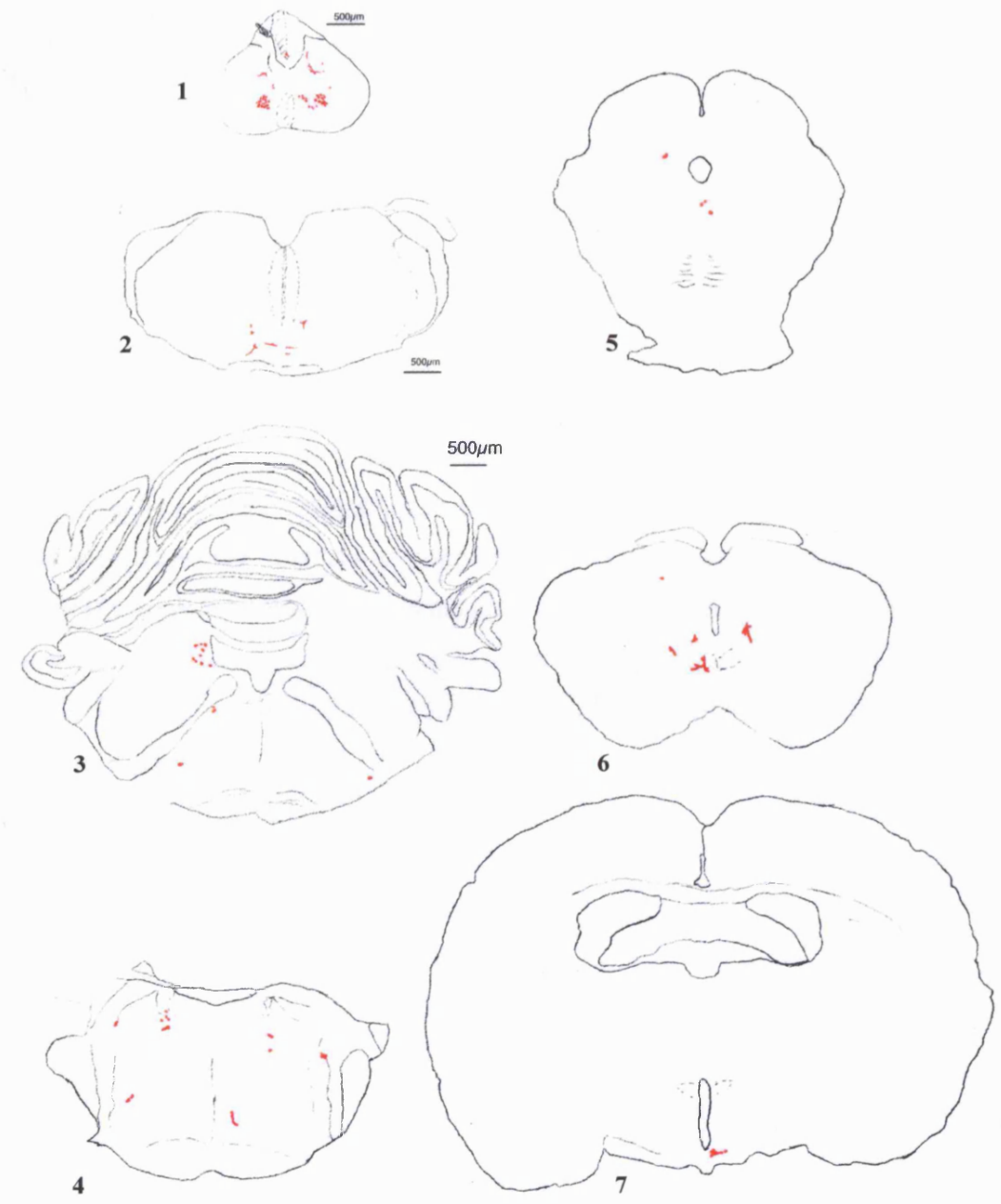


Figure 3.3.3-c: Mapping of HSV1.pR19CMVLacZ expression in the acutely injured SC.

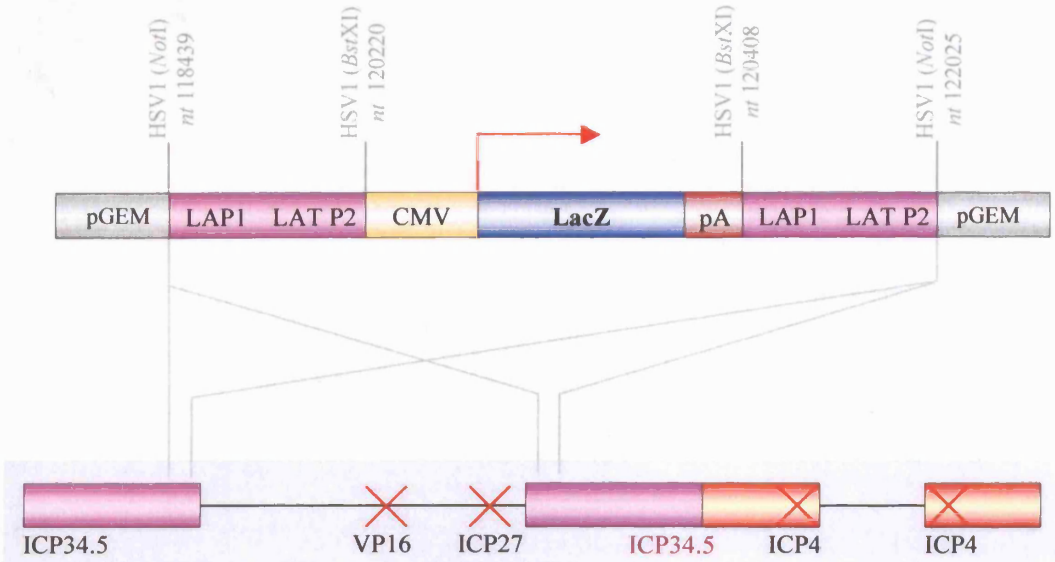
(Group C) Neurons transduced via a single injection in the chronically lesioned CST in the lumber spinal cord. Transduced neuronal cell bodies (represented in red) include: **1:** Ventral and dorsal horn neurons, **2:** Reticular formation neurons **3:** Locus coeruleus, reticular formation and olivary nucleus neurons **4:** Locus coeruleus, trigeminal nucleus and reticular formation neurons **5:** Reticular formation neurons **6:** Central grey and Edinger-Westphal nucleus neurons **7:** Hypothalamic nuclei. Note the similarity of location of the retrogradely transduced neurons to that in Group A, non-injured SC (Figure 3.3.3-a) and in Group B, chronically injured SC Figure 3.3.3-b.

It is important to point out that none of the animals injected with the HSV1.pR19CMVLacZ vector, be it non-lesioned, acutely or chronically lesioned displayed any cerebral cortical neuron transduction. This was an unfortunate discovery in terms of the then envisaged attempts to promote regeneration of the CST (discussed in Chapter 4). Even the direct injection of $2x10^5$ pfu of HSV1.pR19CMVLacZ in the cortex itself resulted in the transduction of only three cortical neurons (shown in Figure 3.3.4-d, panel **A**, page 165). However, this development was not totally unexpected and the potential reasons for this are discussed in sections 3.3.4 and 3.4. At the time that this study was carried out there were no other alternatives that could potentially bypass this problem and the production of neurotrophin expressing constructs was initiated based on this backbone. However attempts at improving the vector backbones continued. As this study drew to a close a new generation of vectors became available. Some of the preliminary experiments that were carried out showed some promise in terms of their ability to target cortical neurons. Some of those results are outlined in the following section.

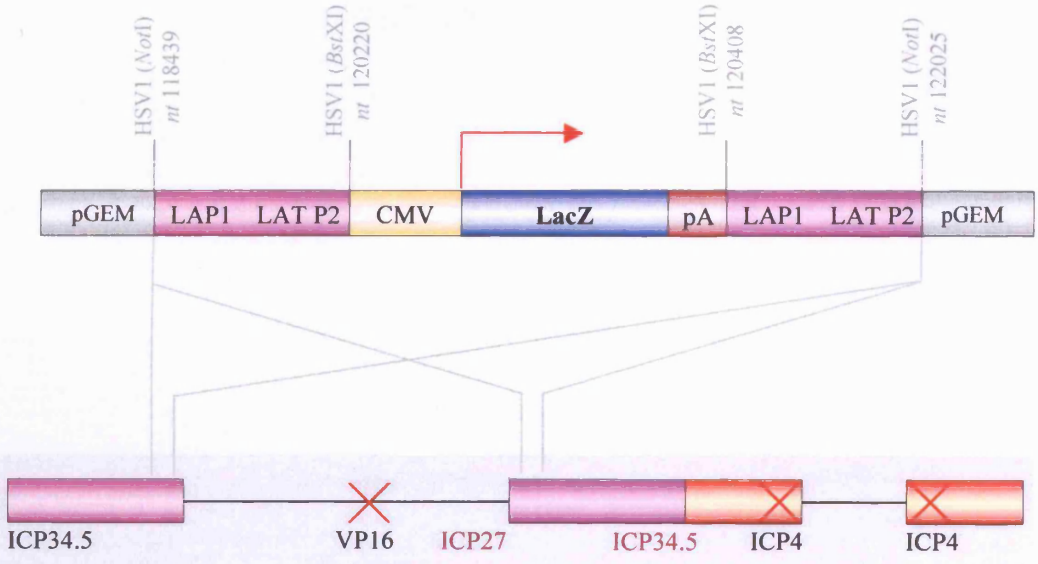
3.3.4 Minimally disabled pR19CMV based vectors

As detailed in the previous section, the highly disabled vector backbone HSV1.pR19CMV was unable to transduce cortical neurons. On the one hand this feature is a testament to the non-toxic nature of this construct (Lilley *et al.*, 2001b) but was also a hindrance in terms of any CST regenerative attempts. However, further vector development carried out in our laboratory opened the possibility of using less disabled vectors to directly transduce those neurons. As this development occurred at the later stages of this study only a few preliminary experiments were carried out. These focused on primarily trying to establish whether they present a more attractive option for the future. Two vectors were considered here: HSV1.RL1$^+$/pR19CMVLacZ and HSV1.ICP27$^+$/RL1$^+$/pR19CMVLacZ (Figure 3.3.1-a, panels **A** & **B** respectively). The two vectors were compared firstly in terms of their ability to sustain the long-term transgene expression in the non-injured spinal cord and secondly on their ability to directly transduce neurons of the motor cortex.

These vectors displayed a growth advantage and could therefore be produced at titres in the order of $2x10^9$ pfu/ml, which is higher than the $5x10^8$ pfu/ml achieved with vector HSV1.pR19CMV. In order to ensure that any differences observed with the two new vectors were not attributed to simply injecting more viral particles, all spinal cords were inoculated with $2x10^6$ pfu, which is higher than the number of particles injected in the HSV1.pR19CMV characterisation experiments. However, it must be emphasised that by the time these injections were carried out the purification of viral stocks and also the experimental parameters had been perfected to improve reproducibility (not presented here), which is likely to have affected overall vector performance. Injections were carried out in non-lesioned animals and at the C6-C7 level (n=3 per time point per vector). LacZ expression was evaluated at two weeks, one and two months post inoculation. The animal with the best transduction selected from each time point is presented in Figure 3.3.4-b for vector HSV1.RL1$^+$/pR19CMVLacZ and Figure 3.3.4-c for vector HSV1. ICP27$^+$/RL1$^+$/pR19CMVLacZ.



A: Vector 1764/ICP27$^-$/ICP4$^-$/RL1$^+$/pR19CMV (HSV1.RL1$^+$/pR19CMVLacZ)



B: Vector 1764/ICP27$^+$/ICP4$^-$/RL1$^+$/pR19CMV (HSV1.ICP27$^+$/RL1$^+$/pR19CMVLacZ)

Figure 3.3.4-a: Schematic representation of RL1+ & ICP27$^+$, HSV1.pR19CMV vectors.

The two vectors depicted above are based on the HSV1.pR19CMV backbone and they have the genes ICP27 and ICP34.5 reinserted in the viral backbone (further described in 3.2.1, page 126).

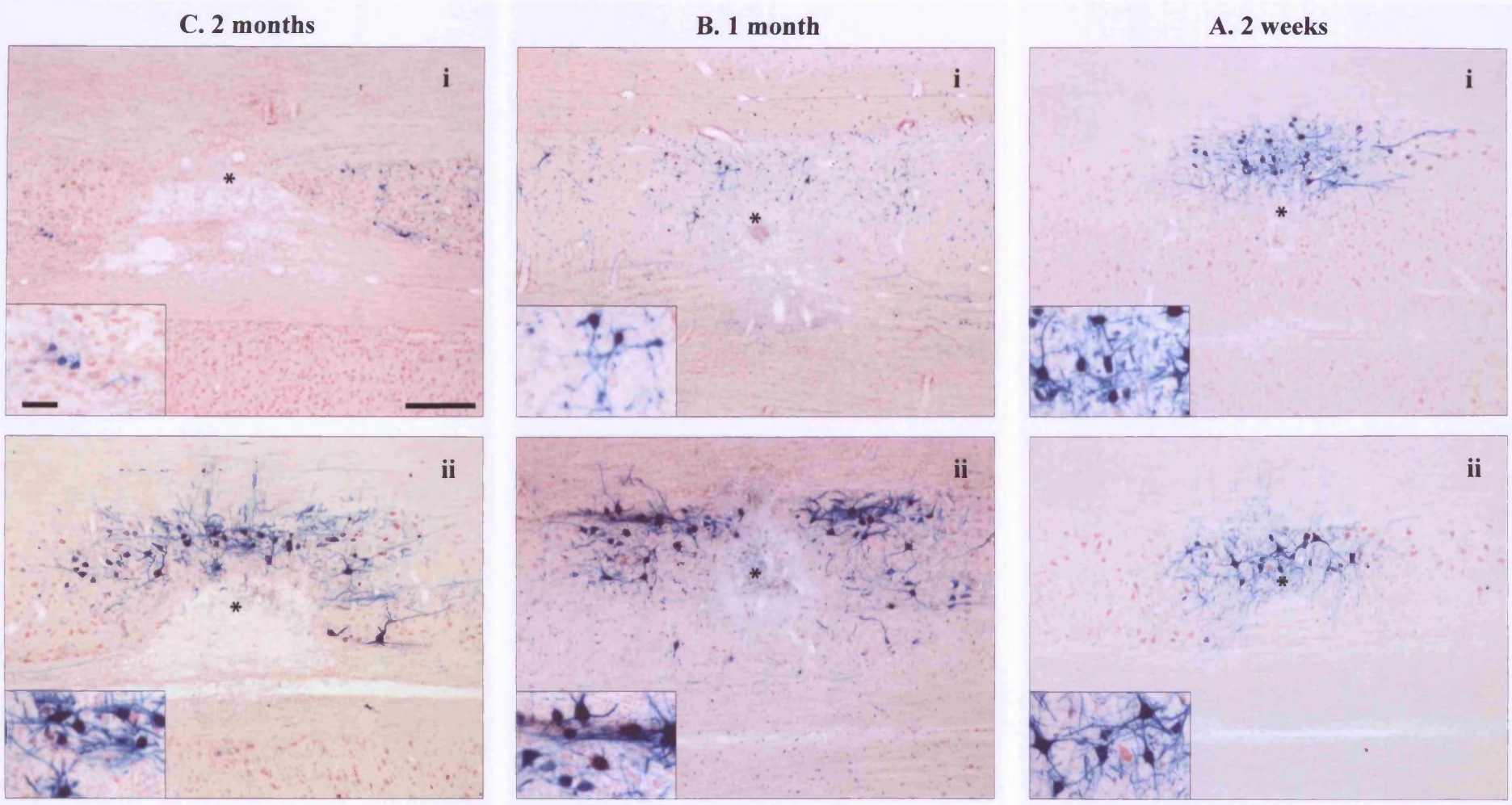


Figure 3.3.4-b: Effect of RL1^{+} reinsertion in HSV1pR19CMV mediated LacZ expression. Longitudinal sections of dorsal (**i**) and ventral (**ii**) spinal cord demonstrate virus mediated transgene expression with ventral motor neurons strongly expressing LacZ. (Injection site *). HSV1.RL1^{+}/pR19CMVLacZ-mediated expression was assessed at the two-week (**Ai & ii**), one month (**Bi & ii**) and two month (**Ci & ii**) time points following a single injection to the cervical spinal cord (C6-C7). High magnification micrographs of transduced neurons are shown in inserts at the lower edge of each photograph. Scale bars: **Ai**=500µm, applies to **Aii**, **Bi**, **Bii**, **Ci**, **Cii**; inset=100µm also applies to all insets.

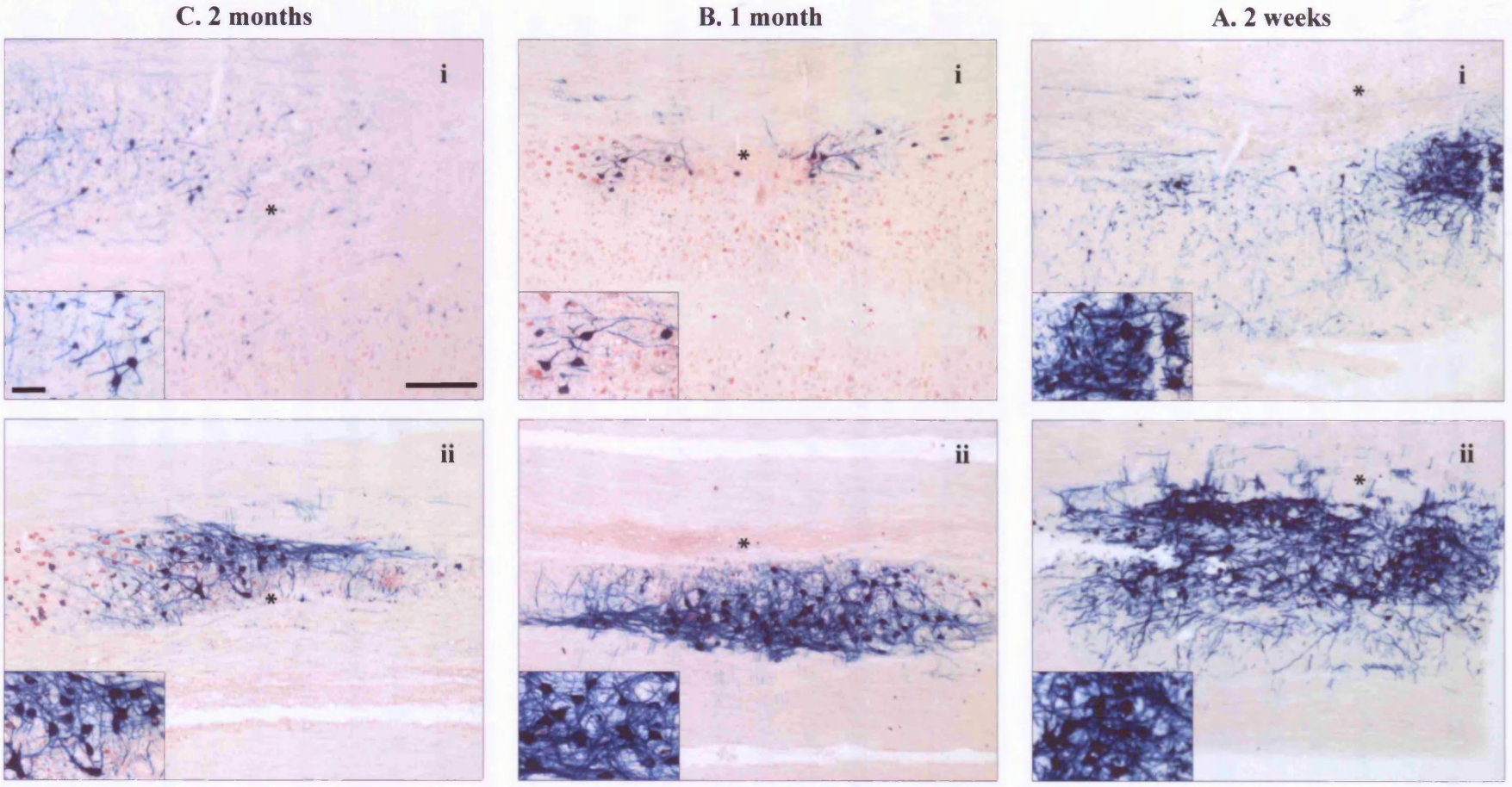


Figure 3.3.4-c: Effect of ICP27 reinsertion in HSV1.RL1^{+}/pR19CMV LacZ expression. Longitudinal sections of dorsal (**i**) and ventral (**ii**) cervical (C6-C7) spinal cord showing vector-mediated expression in motor neurons as assessed at 2-week (**Ai & ii**), one month (**Bi & ii**) and two-month (**Ci & ii**) time points. The addition of ICP27 in the vector enhanced transgene expression in ventral spinal cord at all time points as compared to the same vector without ICP27 as shown in Figure 3.3.4-b. (Injection site *). Scale bars: **Ai**=500μm, applies to **Aii**, **Bi**, **Bii**, **Ci**, **Cii**; inset=100μm, applies to all insets.

For each injection, both ventral and dorsal aspects of the injection site are shown to account for differences in the depth of inoculation that could confound the number of motor neurons transduced. Interestingly, there appears to be little difference between the ability of these two vectors to maintain transgene expression in the long term. As it can be seen by comparing Figure 3.3.4-b and Figure 3.3.4-c, motor neurons transduced with either vector are capable of expressing the transgene for at least two months, which is the longest time point examined. In the case of vector HSV1.RL1$^+$/pR19CMVLacZ there does not seem to be a significant reduction in the number of motor neurons expressing the transgene between the three time points (Figure 3.3.4-b, panels **A, B & C**). However, in the case of vector HSV1.ICP27$^+$/RL1$^+$/pR19CMVLacZ, there is a more apparent, yet subtle and gradual reduction in the LacZ positive neurons between the three time points (Figure 3.3.4-c, panels **A, B & C**).

Overall, inoculation of vector HSV1.RL1$^+$/pR19CMVLacZ appears to lead to the transduction of fewer motor neurons than its less disabled counterpart HSV1.ICP27$^+$/RL1$^+$/pR19CMVLacZ. The number of motor neurons transduced at the two-week time point is significantly higher with the HSV1.ICP27$^+$/RL1$^+$/pR19CMVLacZ vector (Figure 3.3.4-c, panels **Ai & ii**) compared to those transduced at the same time point with vector HSV1.RL1$^+$/pR19CMVLacZ (Figure 3.3.4-b, panels **Ai & ii**). Also, the inserts in each illustration demonstrate that despite the disablement, the transduced neurons appear to have a healthy morphology. Based on the fact that vector HSV1.ICP27$^+$/RL1$^+$/pR19CMVLacZ appeared better than vector HSV1.pR19CMVLacZ and even HSV1.RL1$^+$/pR19CMVLacZ at transducing ventral spinal cord motor neurons, it was decided to examine whether it also performs better in mediating foreign gene expression in cortical neurons. For this experiment, 2.5x10^5 pfu of vector HSV1.ICP27$^+$/RL1$^+$/pR19CMVLacZ (Figure 3.3.4-d, panel **Bi**) or vector HSV1.pR19CMVLacZ (Figure 3.3.4-d, panel **Ai**) were injected directly into the motor cortex of adult rats (n=3 per vector). One-month post injection, the vector mediated transduction of cortical neurons achieved with vector HSV1.ICP27$^+$/RL1$^+$/pR19CMVLacZ is more extensive than that achieved with vector HSV1.pR19CMVLacZ. Even though the actual

number of neurons transduced was not counted accurately, it is evident from the images shown that the difference in the numbers transduced is significant. Ideally, in terms of spinal cord regeneration experiments targeting the CST tract, it would have been more relevant to examine whether CST neurons can be retrogradely transduced following a single spinal cord inoculation with vector HSV1.ICP27$^+$/RL1$^+$/pR19CMVLacZ because it obviously presents a powerful tool for regeneration experiments. As this important development in vector technology came at the closing stages of this study only a few such preliminary experiments were carried out. These produced some very promising results that are discussed briefly in Chapter 6 of this thesis and are relevant not only to the injured spinal cord but also to other tracts of the CNS such as the lesioned optic nerve.

As the vectors assessed here are less disabled they are potentially more toxic than the fully disabled vectors already discussed. It was therefore decided to examine whether either of these vectors were still capable of transducing and maintaining marker gene expression for any length of time in cerebellar purkinje neurons. These particular neurons were selected due to the fact that they are notoriously sensitive to toxic insults. Here they were briefly used to explore whether they are receptive to infection by these vectors and whether expression of the transgene can be supported for any length of time. $2.5x10^6$ pfu of each vector (i.e.: HSV1.RL1$^+$/pR19CMVLacZ or HSV1.ICP27$^+$/RL1$^+$/pR19CMVLacZ) were injected directly into the cerebellum of adult rats (n=3 per vector). LacZ expression was evaluated at the one-month time point and the animals with the best transduction are shown in Figure 3.3.4-d. Even though the number of animals used in this brief experiment was too small to draw any definitive conclusions about their comparative efficacy, it is evident that both vectors have the ability to transduce cerebellar neurons. Both HSV1.RL1$^+$/pR19CMVLacZ (panel **C**) and vector HSV1.ICP27$^+$/RL1$^+$/pR19CMVLacZ appear to successfully transduce purkinje neurons as well as processes of the spinocerebellar tract.

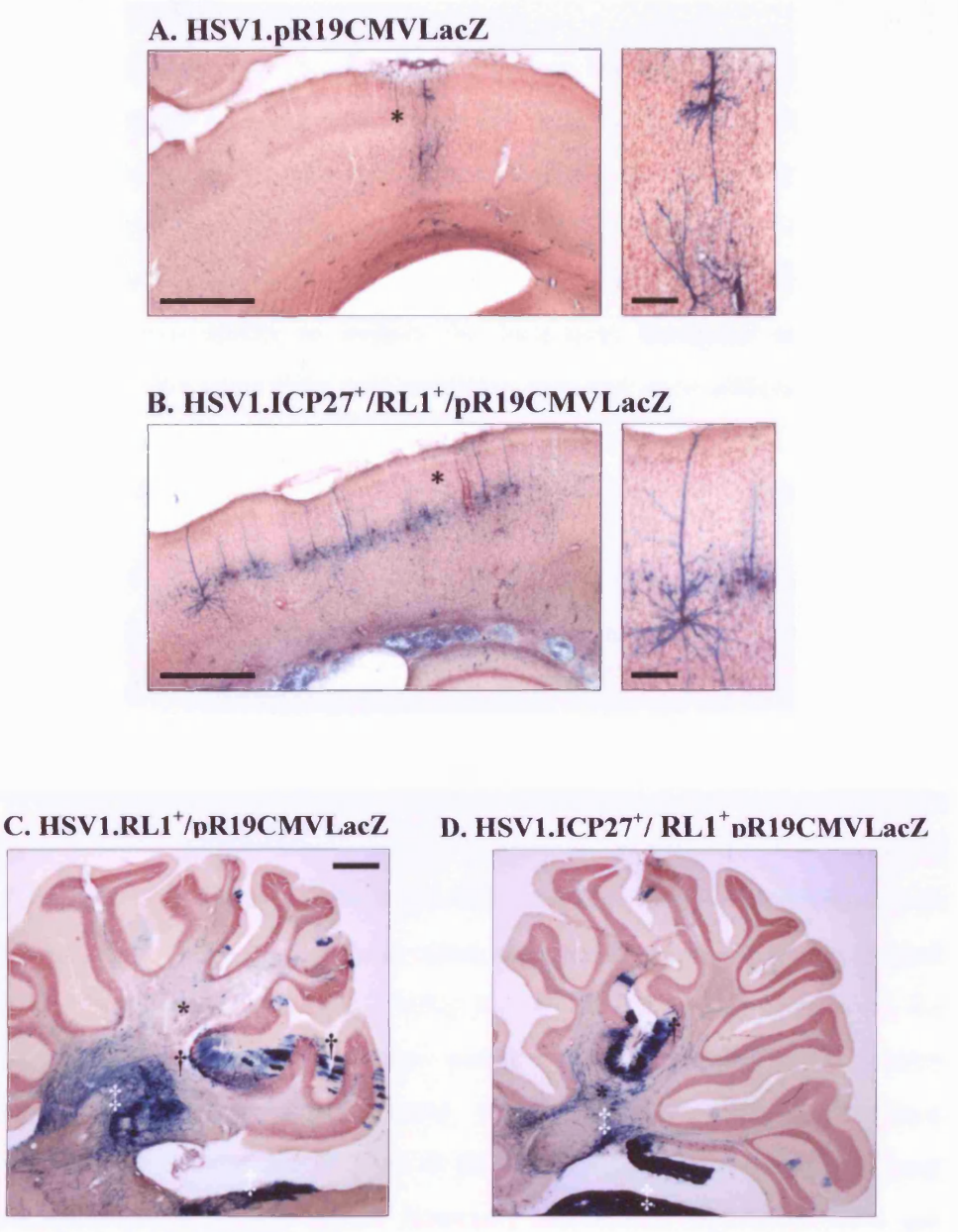


Figure 3.3.4-d: Cortical and cerebellar neuron transduction via pR19CMV backbones.

A, B: Cerebral cortex, low magnification sagittal sections of transduced cortical projection neurons close to the injection site (*) of vector. Note the limited LacZ expression with vector HSV1.pR19CMVLacZ (**A**) but widespread LacZ expression with vector HSV1.ICP27$^+$/ RL1$^+$/pR19CMVLacZ (**B**). Some of the transduced projection neurons are shown at high magnification in the separate images at right. Scale bars: **A**: Left=500μm, Right=100μm; **B**: Left=500μm, Right=100μm.

C, D: Cerebellum, low magnification sagittal sections of cerebellum demonstrating that both HSV1.RL1$^+$/pR19CMVLacZ (**C**) and HSV1.ICP27$^+$/RL1$^+$/pR19CMVLacZ (**D**) constructs appear to transduce purkinje neurons (†) as well as spinocerebellar tract processes (‡) following a single 2.5x10^5 pfu inoculation into the cerebellum. Scale bars: **C**=1000μm, applies to **D**.

3.4 Discussion

The HSV1 constructs described here were designed with the view of creating a platform that could be used to evaluate the potentially therapeutic effect of regeneration-promoting molecules and specifically to evaluate their ability to promote the survival and regeneration of injured CNS neurons. In this Chapter, experiments were designed with the view to screen the available vector constructs for their ability to sustain the long-term transgene expression in neurons while at the same time demonstrating minimal toxic effects in the CNS environment. This is a particularly difficult balance to reach with regards to a sensitive but at the same time demanding tissue in terms of regeneration.

The first construct characterised was the HSV1/P2$^-$.pR20.5*vhs* vector. This vector appeared to be capable of transducing neuronal as well as glial cells following a single inoculation in the spinal cord which is a natural property of the virus (Huang *et al.*, 1992). It could be argued that in this case this noticeable preference for glial cell transduction may be artifactual. It could be a reflection of an injection that accidentally delivered the majority of the virus in white rather than grey spinal cord matter. This is possible as these experiments were carried out in the initial stages of this study when the injection technique and related experimental parameters where still being perfected. The needle used in all the HSV1/P2$^-$.pR20.5*vhs* and only the initial HSV1.pR19CMVLacZ vector experiments, was a flexible silicone needle (SilFlex, World Precision Instruments, Europe) that was thought at the time to induce less neuronal tissue damage. Due to its flexible nature however, stereotactic injections were not accurate enough for this type of experiment. Indeed, the use of this model of injection needle was at a later date abandoned. It quickly became clear that due to its blunt nature, the needle tip induced a compression injury to the spinal cord that could confound the regeneration experiments that followed. Still, as plausible as this explanation may sound, it is unlikely to be the cause of this glial cell preference, as this was not an isolated case. The same expression pattern was observed when HSV1/P2$^-$.pR20.5*vhs* vector was compared to the less disabled vector HSV1.pR19CMVLacZ (Figure 3.3.2-e, page 143). These experiments were the first to be carried out with a standard, rigid stereotactic needle (as

outlined in the methods section). Even though there were now more neuronal cells transduced by the HSV1/P2⁻.pR20.5*vhs* vector, the majority were still glia. The HSV1.pR19CMVLacZ vector displayed a comparatively stringent preference for neuronal cells and clearly maintained the innate HSV1 ability for retrograde transport to neuronal cell bodies distal to the injection site, not seen with the HSV1/P2⁻.pR20.5*vhs* vector.

This pattern of cell transduction has been reported by other researchers working with HSV1 vectors bearing a similar level of disablement (Marsh *et al.*, 2000). In their case, spinal cord organotypic cultures were transduced to express LacZ with a vector that was not as highly disabled as the one used here. Their vector was deleted for ICP4 and carried the same *in*1814 inactivating mutation in VP16 (Ace *et al.*, 1989; Johnson *et al.*, 1994; Holmes *et al.*, 2000). This group microinjected their construct at a high MOI and also found that despite the neurotropism of HSV1, glial cells were also highly transduced to express the transgene, which was under the control of a CMV promoter. Marsh and colleagues (2000) suggested that the lack of neuronal transduction may have been due to the lack of axonal processes in the organotypic cultures. Similar to the vector employed by Marsh *et al.* (2000), vector HSV1/P2⁻.pR20.5*vhs* described here was capable of transducing mainly neuronal cells in addition to a number of supporting cells when tested in organotypic hippocampal cultures (Lilley *et al.*, 2001b). Their explanation is therefore plausible for their organotypic spinal cord culture system and is in line with the expression seen in organotypic hippocampal cultures transduced with the HSV1/P2⁻.pR20.5*vhs* vector (Lilley *et al.*, 2001b). It does not however address the pattern observed in our *in vivo* spinal cord delivery of the HSV1/P2⁻.pR20.5*vhs* vector, where neuronal processes are easily accessible.

A more logical explanation behind this difference may have to do with the levels of ICP0, which this virus potentially expresses (Lilley *et al.*, 2001b). It has been postulated that this may be the reason behind the fact that the HSV1/P2⁻.pR20.5*vhs* vector appears to infect different neuronal cell populations *in vitro* and *in vivo* compared to a less disabled vector such as HSV1.pR19CMV (Palmer

et al., 2000; Lilley *et al.*, 2001b). As already discussed, vectors such as HSV1/P2$^-$.pR20.5*vhs* have some residual levels of ICP0, as they are not deleted for this gene. In this case, the levels of ICP0 may be too low to detect by Western blotting or to cause a microscopically evident toxic effect but the protein may be present in sufficient levels to transactivate the exogenous promoters (RSV and CMV) present in the pR20.5 expression cassette (Lilley *et al.*, 2001b). It is plausible that glial cells have a lower requirement for ICP0 in order for this to occur compared to neuronal cells. This would explain why the less disabled HSV1.pR19CMV vector is seemingly better at transducing neurons *in vivo*. It could also to some extent explain the punctate, peri-nuclear staining observed in neurons transduced by the HSV1/P2$^-$.pR20.5*vhs* vector. This staining pattern was observed only in neuronal somata and not glia and it may be indicative of low level transgene expression (Ho and Mocarski, 1989). This could be explained if ICP0 levels were sufficient to transactivate the CMV promoter in glial but not in neuronal cells. It is very difficult to say with any certainty that it is the small changes in the levels of ICP0 that make such a difference in terms of the vector's ability to transduce glia rather than neuronal cells. Previous researchers in this laboratory have tried to look at the differences in ICP0 expression between the disabled vectors HSV1/P2$^-$.pR20.5*vhs* and HSV1.pR19CMV. This was done using Western blotting techniques, which are perhaps not sensitive enough to detect subtle differences. Nevertheless, there appears to be very little difference in the levels of ICP0 expressed by either vector (Lilley *et al.*, 2001b). However, this pattern of transgene expression and the involvement of ICP0 were perhaps not investigated accurately enough in the *in vitro* comparison experiments to make this claim. This can be said not only because the comparison was made using Western blotting, but also because these experiments were based on the principle that by using equal titres for each vector, it is possible to compare the ICP0 expression patterns for each of these vectors. Both vectors were normalised prior to infection of an equal number of non-complementing, BHK cells. However, in this case this may not have been true. As described previously, the HSV1.pR19CMV vector that contained the exogenous cassette in each of the endogenous LAT regions displayed a growth advantage and could therefore be propagated at higher titres in culture. To put it

simply this means that in the case of the HSV1.pR19CMV vector one viral particle may have corresponded to one plaque forming unit but in the case of the more disabled HSV1/P2$^-$.pR20.5*vhs* vector more than one particle may equate to one plaque forming unit (Lilley, 2000). However, ≈150 molecules of ICP0 are packaged into the tegument region of each purified viral particle (Yao and Courtney, 1992). It is therefore possible that there was more ICP0 protein delivered to the infected cells in the case of vector HSV1/P2$^-$.pR20.5*vhs* as more particles would be needed to achieve an equal titre to that of HSV1.pR19CMV vector. This would mean that there could potentially be some confounding in the results obtained, as some ICP0 would be delivered rather than produced *de novo* from the vector itself. Overall, the experiments designed by Dr C.E. Lilley to detect small differences in IE gene expression were relatively too crude considering the subtle differences in ICP0 that they set out to detect. Since no further investigation has been carried out into the levels of ICP0, it is not possible to draw any definite conclusions with regards to the role of ICP0. It would be interesting to see whether normalising the two viruses for the number of viral particles actually makes a detectable difference in the levels of ICP0.

Another point that becomes apparent from the comparison of the HSV1/P2$^-$.pR20.5*vhs* and HSV1.pR19CMV vector backbones is that there is a noticeable amount of inflammation around the lesion site. Injections with HSV1/P2$^-$.pR20.5*vhs* almost invariably result in the presence of a central area of necrosis just surrounding the injection tract. This is easy to see, both in the case of the GFP (Figure 3.3.1-b, panel **Ai**) and LacZ (panel **Bii**) stained section. The area of inflammation can be identified by the presence of a collection of cellular debris. These appeared to be mononuclear infiltrate cells, which would indicate the presence of an inflammatory reaction. Interestingly, the same pattern was noted in the early experiments carried out with vector HSV1.pR19CMVLacZ as demonstrated in Figure 3.3.2-e (panel **Bii**, page 143). In the later case, the area of necrosis was not diffuse but it appears to be prominent. However, later experiments with this vector demonstrated a less prominent reaction around the lesion site. Both these vectors had previously been reported to be non-toxic to embryonic DRG or adult hippocampal cultures, even at high MOIs (Lilley *et al.*,

2001b). Moreover, they have been shown to express minimal amounts of IE genes such as ICP22 and ICP47 which if present, can cause host toxicity (Lilley *et al.*, 2001b). It is therefore logical to suggest that the reaction observed in the early experiments described here may have been due to the mechanical damage inflicted to the spinal cord during the injection procedure, rather than a process directed against the vector itself. As discussed already, the injection technique employed in the early experiments did cause more tissue trauma than that observed in later experiments. Alternatively, the then viral stock production procedure was less refined than that employed in the regeneration experiments reported in later chapters. For example, the initial stock production of the HSV1/P2$^-$.pR20.5*vhs* and later HSV1.pR19CMVLacZ vectors did not include a filtration step. Of course, both these vectors were propagated in specially designed complementing cell lines (such as the 27/12/M:4 described in the methods section in Chapter 2). Western blots have demonstrated that complementing cell lines produce large amounts of ICP0, ICP22 and ICP47 (Thomas *et al.*, 1999; Lilley *et al.*, 2001b). Even though cell line products and debris were in theory removed from the virus stock during stock concentration and purification step (Chapter 2), the method employed at the time was not as stringent and contamination with such products could not be excluded. This would allow for the possibility that the high titre stocks used here may have been causing a local inflammatory reaction. This appears even more plausible when one considers the alternative way that these vectors have been used to transduce spinal cord motor neurons. Specifically, when the HSV1.pR19CMVLacZ vector was delivered to spinal cord motor neurons via a sciatic nerve injection, there was minimal presence of cell infiltration around the transduced ventral horn spinal motor neurons, hinting to the possibility that these backbones may not be immunogenic (Palmer *et al.*, 2000). This point becomes more evident when one considers the sections displaying the injection sites of either HSV1.RL1$^+$/pR19CMVLacZ or HSV1.ICP27$^+$/RL1$^+$/pR19CMVLacZ (Figure 3.3.4-b and Figure 3.3.4-c respectively), which would be expected to have a higher level of toxicity since they still retain the ICP34.5 or/and ICP27 genes. However, the injection site of these two vectors does not appear to be that much different in terms of an inflammatory response compared to the more disabled

HSV1.pR19CMVLacZ vector. This would point more towards the potential implication of stock purity in terms of local tissue necrosis around the inoculation site. Finally, given the fact that β-Gal is a large, bacterial protein it too in theory has the potential to cause an immune reaction. Throughout the experiments carried out by this operator as well as others in this lab, no differences were observed in terms of morphology at the lesion site when a GFP expressing vector was used. However, this is something that could have been examined in greater detail using suitable immunological markers. This had not been previously performed and was not formally performed in this study.

Based on the fact that the vectors described here are disabled for different gene combinations, it is logical to examine the ways in which the nature of these deletions impact on their expression pattern as well as their ability to induce an immune reaction from the host. Thinking along these lines, it is possible to put forward another reason as to why there appears to be a prominent local reaction around the injection site and also why the expression achieved with the HSV1/P2$^-$.pR20.5*vhs* vector appears to be so short lived. Essentially, the major difference between the HSV1.pR19CMV vector and HSV1/P2$^-$.pR20.5*vhs* is the location of the cassette. As discussed previously, the ectopic location of the pR20.5 cassette (disrupting the *vhs* gene) appeared to be play a role in the ability of the vector to sustain the expression of transgenes in the long term (Palmer *et al.*, 2000; Lilley *et al.*, 2001b). The function of the *vhs* gene is to shut down host protein synthesis of molecules that can promote an immune response such as interleukin 1 (Suzutani *et al.*, 2000), interferon (Lin *et al.*, 2004) and MHC Class I and II (Hill *et al.*, 1994; Trgovcich *et al.*, 2002) in neuronal cells (Barzilai *et al.*, 2006). Therefore the host's immune system could react more readily to the infected cells resulting in the local reaction as well as the loss of a number of transduced cells. This could confound the overall expression picture of the transgene. The above possibility could also explain the lack of any morphologically significant tissue reaction to the inoculum in the case of the less disabled vectors. This could be due to the fact that the ICP34.5 protein contributes to the immune evasion of vector infected cells by suppressing INFβ (Pasieka *et al.*, 2006) and MHC Class I and II production (Trgovcich *et al.*,

2002) from infected cells. These less disabled vectors do have the ICP34.5 protein and can essentially suppress the ability of the cell to initiate a local reaction. Furthermore, this could explain why these vectors appear to be able to sustain transgene expression for at least two months post-inoculation. It is possible that the transduced cells are not cleared from the host. That is not to say that the HSV1.RL1^{+}/pR19CMVLacZ or HSV1.ICP27^{+}/RL1^{+}/pR19CMVLacZ vectors do not display any toxicity to the cells they transduce. As these vectors were never used in any regeneration experiments they were not further characterised neither by this author nor by the operator that produced them as part of a separate project. As a consequence, there are no data that detail their IE gene expression patterns or their behaviour *in vitro* or *in vivo*. This is something that should undoubtedly be addressed prior to the application of these vectors in any future CNS regeneration experiments.

Preliminary results illustrated that the HSV1.pR19CMV vector, is overall a more promising tool for gene delivery to the CNS and spinal cord neurons in particular. A single vector inoculation at the C6-C7 level results in a stable and efficient transduction of motor neurons in the ventral horn and sensory neurons in ipsilateral DRGs that project to the immediate vicinity of the injection site (Figure 3.3.2-d, page 141). Transduced neurons strongly express the marker gene for at least 21 days, which was the longest time point tested here. The fact that vector HSV1.pR19CMV was unable to transduce cortical neurons at a potentially significant level, either by direct cortical injection (Figure 3.3.4-d, panel **A**) or by retrograde transport, was a hindrance in the then envisaged regenerative studies, especially as one of the main pathways considered for regenerative attempts was the CST. In earlier experiments of other research groups it was suggested that the difference in the expression patterns of LacZ following intracranial inoculation vary depending on whether the vector is replication-attenuated, as in the case of an ICP0 deleted vector, or replication-incompetent, like the vectors utilised here (Chiocca *et al.*, 1990). Interestingly, this feature appears to be a reflection of the non-toxic nature of the HSV1.pR19CMV vector, a notion that is supported in the later experiments conducted in this laboratory. Comparing the expression patterns of vectors

carrying different levels of disablement after a single striatum inoculation it becomes evident that the more severe the disablement, the less able the vector is to transduce cortical neurons (Chiocca *et al.*, 1990). Analytically, a single striatum injection of a 17^{+}/ICP27^{-} virus led to the transduction of both striatum and cortical neurons that persisted for 1 month post injection (Lilley *et al.*, 2001b). This pattern resembles the pattern of LacZ expression achieved with a virus that has been deleted for the endogenous ICP0 gene (Chiocca *et al.*, 1990). On the contrary, the striatum injection of HSV1.pR19CMV resulted in the strong transduction of striatal and substantia nigra neurons but transgene expression in transduced cortical neurons appeared to decline sharply after the three day time point (Lilley *et al.*, 2001b). The expression pattern noted in this case resembled that achieved with ICP4 deleted vectors (Chiocca *et al.*, 1990; Maidment *et al.*, 1996). It could therefore be suggested that due to their higher toxicity, less disabled vectors can perhaps overcome the strong inhibition to viral gene transcription which may be present in cortical neurons (Lilley *et al.*, 2001b). This could explain why the less disabled vectors examined here appear to lead to a more efficient transduction of cortical neurons.

However plausible the above explanations may seem, they are unlikely to be the sole reason for the loss of transgene expression, especially in the case of cortical neurons where this occurs so quickly after infection. Early experiments designed to examine the toxicity of mutants carrying different mutations in IE genes were able to shed some light in their ability to establish a latent infection in host cells. Even though the immune system does play a role in terms of the longevity of transgene expression (also reviewed in detail in Chapter 7), the apparently quick loss of transgene expression in transduced cortical neurons is more likely to be due to the repression of promoters rather that the clearance of virally infected cells. This statement emanates from the culmination of evidence from research carried out by this as well as other laboratories. The experiments exploring any potential silencing occurring with our fully disabled vectors in cortical neurons, are based on the early experiments by Samaniego *et al.* (1998). Briefly, it was demonstrated that the silencing of exogenous promoters in a highly disabled virus is attributable to the lack of expression of ICP0. Providing ICP0 can re-

stimulate the expression of the transgene that is controlled by the exogenous promoter (Samaniego *et al.*, 1998). Further experiments carried out in our laboratory (non- published data), confirm that indeed the loss of expression from cortical neurons so quickly after injection is most likely to be due to the repression of transgene expression rather than the immune mediated clearance of the vector infected cells.

Firstly, it was demonstrated that two weeks following a striatum injection of $5x10^5$ pfu of vector HSV1.pR19CMVLacZ, there may be no LacZ staining but a large number of cortical neurons can be found to harbour copies of LAT mRNA (performed by Dr J. Palmer and included here as reference, non-published material).

Cortex (†) & Substantia Nigra (‡)

The hybridised, sagittal sections shown above, demonstrate the presence of LAT mRNA (black staining) in cortical neurons two weeks after HSV1.pR19CMVLacZ vector inoculation. Their presence signifies that the viral genome is present in cortical neurons but the CMV promoter governing the expression of the LacZ marker gene is silenced (Dr C.E. Lilley – personal communication). A different study carried out in parallel to the one described here, attempted to replicate the Samaniego *et al.* (1998) experiments in order to prove that it is the exogenous promoter controlling the transgene that is silenced and transgene expression can be re-initiated using a vector that is not as highly disabled. The striatum of adult rats was injected with the fully disabled vector HSV1/P2$^-$.pR20.5*vhs* and as expected, the LacZ and GFP expression ceased within three days. Two weeks later, the same animals were re-infected with vector 17$^+$/ICP27$^-$, which carried no marker gene. The cortical neurons were now

able to re-express the LacZ marker gene. Of course this could only have been produced if the initial construct was still present within the transduced cells. The above experiment supports the notion that the Samaniego principle applies to our vectors *in vivo* and can potentially explain the lack of transgene production in these neurons (this work was carried out by Dr C.E. Lilley as part of a separate research project and is included here as a reference). This is a suitable point to bring up another important issue that was considered at the time these experiments were carried out. The marker gene used for most of the experiments designed to evaluate the length of transgene expression was βGal. One of the criticisms that this work has received in the past is that the choice of this transgene may have, to some extent, confounded the outcome of these studies. This is based on the fact that the βGal protein is a very stable protein with a long half-life of ≈43 hours, at least in human fibroblasts (Ko *et al.*, 1983). Due to this long half-life the LacZ staining can be detected for a very long time and indeed some reports suggest that the use of histological staining techniques, such as those used here, can not be used reliably to evaluate the activity of the exogenous promoter driving its expression at the early stages of latency establishment (i.e.: the first twenty one days post transduction) due to the persistence of the existing protein rather than the production of new molecules (Margolis *et al.*, 1993; Lokensgard *et al.*, 1994). The normalising factor would be that there are obvious differences in between the vectors examined here which would be lost if this was the case. It therefore follows that even if there is an element of confounding, then this is uniform enough to allow differences between vector constructs to be manifested. More importantly, it should be emphasised that the transgene expression obtained by the least disabled vectors, was assessed at the two month time point by which any LacZ staining present should represent a new and *de novo* synthesis of β-galactosidase (Lokensgard *et al.*, 1994).

Despite the lack of significant neuronal transduction observed with vector HSV1/P2$^-$.pR20.5*vhs* (Figure 3.3.1-b, page 136) as well as its inability to support long-term transgene expression, at the time, it presented the best practical compromise between cytotoxicity and *in vivo* transduction. Given the lack of alternatives it was decided to proceed with the production of neurotrophin

expressing vectors based on this backbone. A panel of vectors expressing NT3, BDNF or CNTF in combination with each other or with the marker gene for β Gal were constructed and characterised (not presented here). However, due to the inability to produce high titre stocks consistently, as well as the lack of long-term transduction, it was impossible to draw any firm conclusions with regards to the ability of HSV1/P2$^-$.pR20.5*vhs* vector-mediated transduction to influence axonal regeneration. With hindsight, it might have been more prudent to accept the fact that the HSV1/P2$^-$.pR20.5*vhs* backbone was not suitable as a tool for regeneration studies targeting the spinal cord and not proceed to the construction of neurotrophin expressing versions at that point. Admittedly, as the production of the better-suited HSV1.pR19CMV backbone was the subject of another research project there were not many alternatives available at the time. It would perhaps have been more productive to instead concentrate efforts on first perfecting other parameters that could affect the outcome of regeneration studies such as improvements in stock purification protocol and addressing the lack of reproducibility in the *in vivo* spinal cord lesioning model or vector delivery method. This was instead done in parallel with vector construction but admittedly not in a methodical manner.

SUMMARY

The experiments performed in this chapter demonstrate two points. Firstly, widespread and more prominent transgene expression is achieved by placing the expression cassette within the endogenous LAT region, as is the case with the pR19CMV cassette. Secondly, it was shown that a maximally deleted vector backbone is less efficient at transducing neuronal cell populations compared to other minimally disabled constructs, thus demonstrating that some viral genes are involved in mechanisms that favour longer transgene expression

The production of the HSV1.pR19CMV backbone was the first of the highly disabled HSV1 constructs that retained the innate ability of HSV1 for retrograde intra-axonal transport (Bak *et al.*, 1977) while maintaining a high level of transgene expression (Lilley *et al.*, 2001b), as shown here. The creation of this construct presented the first realistic and exciting opportunity to modify the

injured CNS milieu. The transduction achieved with this vector, even in considerably distant sites projecting around and into the lesion site, is strong and more importantly, can be maintained for a length of time that could potentially affect regeneration studies (Figure 3.3.3-a, b and c). In addition, transduction of different neuronal subtypes achieved with this vector is not affected by the presence of a lesion in the spinal cord. These features offered a major advantage in terms of future regeneration experiments and presented a formidable tool that could potentially allow us to explore the function of regeneration related molecules targeting tracts such as the CST (Chapter 4), RST (Chapter 5) and optic nerve tract (Chapter 6) in axotomising injury paradigms. The chapters that follow describe the steps taken to construct neurotrophin expressing vectors based on the HSV1.pR19CMV backbone with the view to explore the potential of these molecules to influence regeneration in each of these systems.

CHAPTER 4.0

VECTOR-MEDIATED DELIVERY OF NEUROTROPHIN-3 IN THE INJURED CORTICOSPINAL TRACT

4.1 Introduction

Damage to the long descending tracts of the spinal cord such as the corticospinal tract (CST), is not followed by any meaningful regeneration, in contrast to the response exhibited by injured PNS axons. The CST shows very limited sprouting following transection (Bregman *et al.*, 1989; Schnell *et al.*, 1994) and a number of technically varied attempts have managed to induce only a limited degree of regeneration. Unlike some other tracts of the CNS which can regenerate into segments of peripheral nerve grafts (Richardson *et al.*, 1980; David and Aguayo, 1981; Berry *et al.*, 1986; Morrow *et al.*, 1993), CST axons, are notoriously resistant to even this regenerative paradigm (Richardson *et al.*, 1982; Ye and Houle, 1997; Blits *et al.*, 2000). The particular reasons governing this reluctance of CST fibres to regenerate, along with a review of the different attempts made at instigating their regeneration and maximising their potential are further examined in the introductory segments of this chapter.

Amongst the different approaches applied, neurotrophic factor delivery has been extensively studied for its ability to promote regeneration of axotomised CST fibres (Lacroix and Tuszynski, 2000; Jones *et al.*, 2001; Sayer *et al.*, 2002; Blesch *et al.*, 2002). Neurotrophin 3 (HDNTF3 or NT3) in particular has been shown to be a promising growth-augmenting molecule (Schnell and Schwab, 1993; Schnell *et al.*, 1994; Tetzlaff *et al.*, 1994; Grill *et al.*, 1997a; Bradbury *et al.*, 1998; Sayer *et al.*, 2002; Zhou *et al.*, 2003; Hagg *et al.*, 2005). As it was demonstrated in Chapter 3, highly disabled HSV1 vectors and vector HSV1.pR19CMV in particular (Lilley *et al.*, 2001b), can efficiently support the expression of transgenes in both the lesioned and chronically lesioned CST with minimal cytotoxic effects. Hence, it presents a suitable means of delivering NT3 since a single injection of the vector would ensure neurotrophin production for at least 1 month in transduced neurons. Unfortunately this vector cannot directly transduce CST neurons.

The experiments outlined in this study set out to establish whether delivering NT3 to a chronically lesioned spinal cord by means of a highly disabled HSV-1 vector have a positive impact on CST axonal regeneration. Additionally, and based on well documented reports that embryonic E14 spinal cord segments

transplanted within the lesion site are capable of inducing regeneration of CST fibres in the presence of biologically active NT3 (Schnell and Schwab, 1993; Bregman *et al.*, 1997b), the ability of HSV1.pR19CMVrNT3 vector to mediate a comparable effect is also investigated. Finally, a brief study on the ability of a new generation of minimally disabled vectors to transduce CST neurons via either spinal cord or brain inoculation is outlined.

4.1.1 Anatomy of the corticospinal tract (CST) in the rat

Even though in lower mammals such as rodents, the location of the CST is anatomically different to that of primates (Lacroix *et al.*, 2004), it has been extensively targeted for experimental paradigms focused on regeneration. One reason for this is the prime importance of this tract in carrying information regarding fine and precise movements. It is the single longest axonal pathway within the mammalian CNS and a schematic diagram of its overall location is shown in Figure 4.1.1-a. In rats, most CST fibres originate from the primary motor cortex (Fr1 and Fr3) (Tracey, 1994) with only a small proportion originating from the sensory cortex (Wise *et al.*, 1979; Tracey, 1994). The CST fibres in the adult rat originate from the cell bodies of pyramidal neurons located either in layer V of the motor cortex, or to a lesser extent, in layer Vb of the primary sensory cortex (Hicks and D'Amato, 1968; Miller, 1987; Joosten, 1997; Brosamle and Schwab, 2000). Once generated around embryonic day 15 (E15), (Berry and Rogers, 1965; Miller, 1987), neurons migrate to their final location in the cortex and initiate their axonal elongation. From layer V or Vb, axons pass through the internal capsule and cerebral peduncle to the ventral pons and on top of the pyramids in the medulla. CST axons reach the caudal medulla about the time of birth and then decussate (Tracey, 1994). On route, only 2% of CST fibres give off collaterals to other nuclei such as the red nucleus, pontine nuclei and the olivary complex (Akintunde and Buxton, 1992). From then on, the main tract crosses the midline and runs in the base of the dorsal columns (Tracey, 1994). In general, fibres originating from pyramidal neurons located in the forelimb areas of either sensory or motor cortices project to the cervical enlargement and those originating from neurons of the hind-limb area terminate in the lumbar enlargement of the spinal cord (Li *et al.*, 1990; Tracey, 1994).

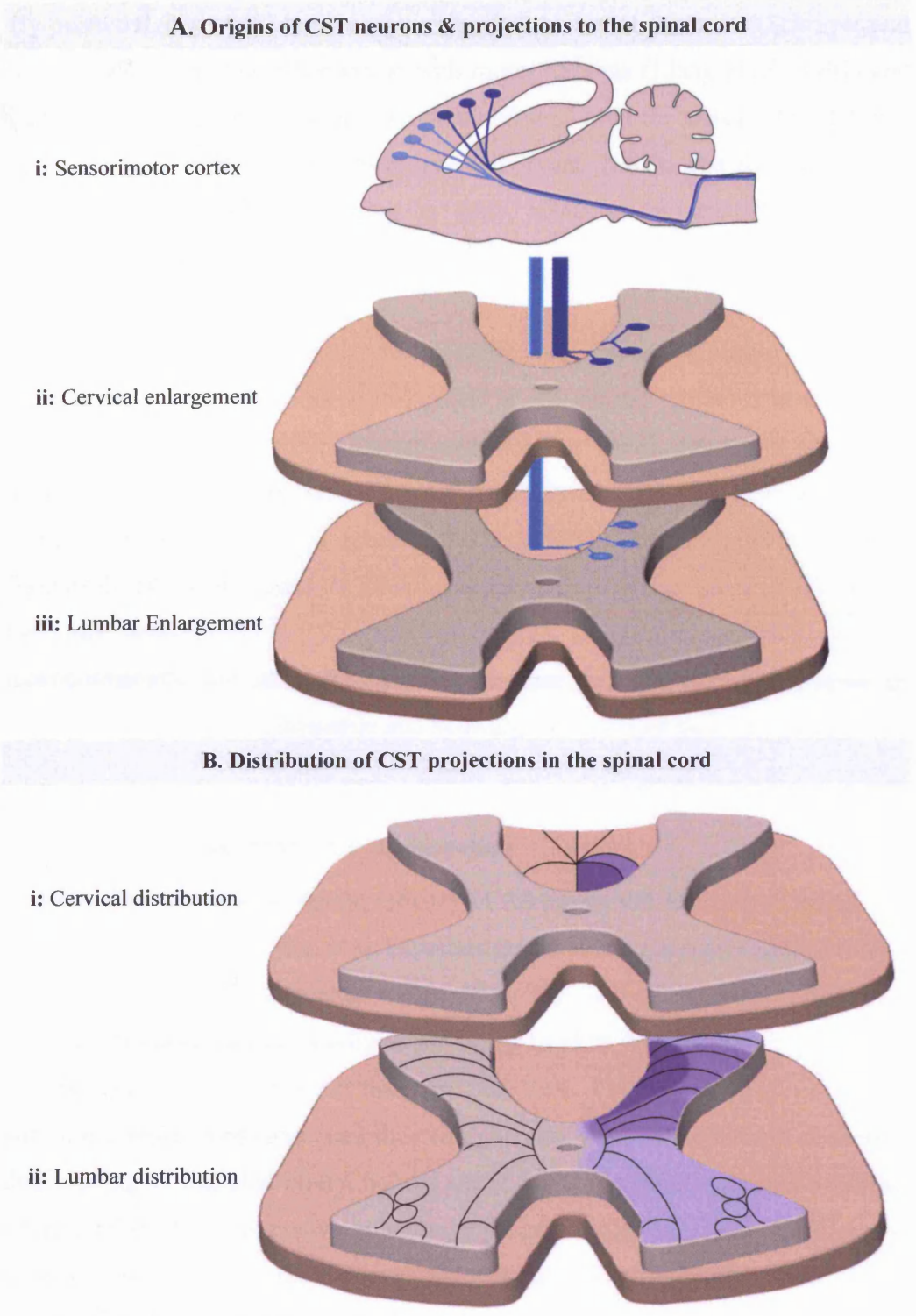


Figure 4.1.1-a Projections of the CST in the rat.

A: CST fibres originate from the sensorimotor cortex (**Ai**) and project to the spinal cord after decussating in the dorsal medulla. The main CST projects to the grey matter with the more dorsal neurons projecting to the cervical enlargement (**Aii**) while the lumbar enlargement receives projections from the more ventrally situated pyramidal neurons (**Aiii**). **B:** The distribution of CST fibres within the cervical (**Bi**) and lumbar (**Bii**) spinal cord enlargements is designated by increasing colour intensity reflecting denser projections in that area. (Adapted from: Tracey 1994 and Joosten 1997).

By postnatal day 14 (P14) fibres have reached the sacral segments (Schreyer and Jones, 1982). Terminal arborisation with motor neurons (Liang *et al.*, 1991) and interneurons within the grey matter is initiated soon after the arrival of CST fibres at each spinal cord segment (Schreyer and Jones, 1982). The final branching pattern and varicosity formation is fully achieved by day P28 (Karimi-Abdolrezaee *et al.*, 2002).

Some CST fibres do not cross contralaterally but instead continue their route ipsilaterally, either within the dorsomedial or the ventral funiculus in the white matter (Joosten *et al.*, 1987; Joosten and Gribnau, 1988; Joosten *et al.*, 1992; Joosten *et al.*, 1993; Brosamle and Schwab, 1997; Brosamle and Schwab, 2000). It was originally questioned whether the ipsilateral ventral projection had any functional significance past the developmental stages (Joosten *et al.*, 1992) but it has since been shown that this pathway projects throughout the spinal cord, is morphologically similar to the main dorsomedial CST and may be involved in fine movement control (Brosamle and Schwab, 1997; Brosamle *et al.*, 2000).

4.1.2 Assessment of CST regeneration

When it comes to evaluating the efficacy of the numerous attempts to induce the regeneration of CST axons, it is important to be able to separate spared axons from local sprouting and genuine regeneration. This is not always straightforward as different experimental paradigms are being used as CST models. Axons are flexible and a lot more resilient than they may look. They are not rigid structures within the spinal cord tissue and they can potentially be displaced and stretched, thus eluding an intended injury. Spared axons could potentially interfere with the interpretation of a regenerative outcome, especially those that follow a minimally invasive approach that often leaves the ventral CST component intact. Such lesions include: incomplete dorsal hemisections that fall short of totally ablating the dorsal columns and lateral hemisections that result in a unilateral lesion. Steward *et al.* (2003) proposes that in order to confirm the origin of regenerating fibres, the criteria shown below should be applied:

Criterion	Abbreviation
I	The axon extends from the CNS tissue/lesion interface into the glial scar.
II	In the case of a graft/transplant being used, the regenerating axon can be seen entering and extending into the foreign tissue.
III	The axon originates at or adjacent to the CNS tissue/lesion interface.
IV	The axon follows an uncharacteristic route through the tissue.
V	The axonal regeneration observed does not extend further than what can be justified according to regeneration rates.
VII	The axonal morphology is unlike that accepted for CST neurons (random branching).

Table 4.1.2-a: Criteria for the assessment of regenerating CST axons.

The above criteria are shown here for consideration in relation to the CST regenerative studies as lesioning of the descending component is subject to such variability and were based on a review by Steward *et al.* (2003). These criteria apply to all regenerative studies and they have been referred to throughout the regenerative studies described in this thesis.

It therefore follows that reports of any functional recovery following partial lesions or lesions that leave the ventral component of the CST intact, could reflect mechanisms other than axonal regeneration. These mechanisms can involve functional compensation from spared pathways such as the rubrospinal tract (RST) that is also involved in fine movement control (Whishaw *et al.*, 1990) or stimulation of the central pattern generators (CPGs) by serotonergic sprouting in the distal portion of the lesioned spinal cord. CPG is the collective term attributed to the networks of spinal neurons that are involved in locomotion. Normally, their actions are controlled and coordinated by the brainstem and the thalamus in response to peripheral stimuli. In case of injury, CPGs can act independently and allow some locomotion to take place (Bear *et al.*, 2001), although coordination between forelimbs and hind-limbs is lost. A recent study has demonstrated that after a T11 spinal cord transection and transplantation of foetal raphe cells, segmental spinal cord plasticity contributes to weight support and bilateral foot placement (Ribotta *et al.*, 2000). Therefore, local plasticity is a factor that should always be considered when evaluating reports of CST regeneration accompanied by recovery of motor function.

4.1.3 Responses of CST neurons to axotomy

The response of CST fibres and the chronological events preceding the formation of the glial scar within the lesion site as well as the type of cells involved are not dissimilar to those of other CNS tracts and have been examined in the main introductory Chapter of this study (Chapter 1). However this morphological similarity is accompanied by subtle differences at the molecular level that, to some extent, explain the low regenerative capacity of the CST compared to other CNS tracts. In adult rats, unlike the case in neonates (Bregman *et al.*, 1989), axotomised CST axons do not regenerate following a complete CST lesion that encompasses both the dorsal and ventral components of the pathway (Bernstein and Stelzner, 1983; Kuang and Kalil, 1990; Z'Graggen *et al.*, 2000; Weidner *et al.*, 2001). Plasticity to the injured CST is however conferred if the ventral component of the tract is spared. Transection of the dorsal CST following a unilateral lesion is followed by spontaneous sprouting from injured ventral CST axons which can cross the midline in an attempt to compensate for lost connections (Weidner *et al.*, 2001).

Dorsal CST fibres are capable of very low level sprouting following axotomy but left unaided, growth arrests at the borders of the lesion site before any regenerative attempt is aborted (Bregman *et al.*, 1989; Schnell *et al.*, 1994) and end-bulbs make their appearance on retracting fibre tips (Ramon Y Cajal, 1928; Fishman and Kelley, 1984; Fishman and Kelly, 1984). Similarly there is considerable sprouting of the cut dorsal CST, proximal to a unilateral lesion. A mid-thoracic dorsal hemisection leads to axons arising from the dorsal CST at the cervical level to enter the grey matter where they form functional contacts with propriospinal motor neurons (Bareyre *et al.*, 2004).

4.1.3.1 Gene expression in lesioned CST neurons

The intrinsic capacity of CST neurons to regenerate is a reflection of their ability to initiate the production of multiple growth-associated proteins (Anderson *et al.*, 1998; Anderson and Lieberman, 1999) and sustain the expression of more than one of these molecules (Bregman *et al.*, 1989; Mason *et al.*, 2000; Bomze *et al.*,

2001) long enough for them to overcome the inhibitory nature of the injured spinal cord environment (Fawcett and Asher, 1999; Sandvig *et al.*, 2004). The gamut of proteins expressed following a CST lesion depends on the location of the lesion itself. Firstly, GAP43 transcription is up-regulated if axotomy is inflicted sub-cortically (Tetzlaff *et al.*, 1994) but not if the lesion is at the level of the brain-stem (Kalil and Skene, 1986; Reh *et al.*, 1987). Secondly, more prominent axonal sprouting is observed after a lesion is exacted sub-cortically compared to when it is inflicted in the medullary pyramids or spinal cord dorsal columns (Fishman and Mattu, 1993). In another, more detailed and extensive study it was demonstrated that mRNA levels of the axotomy-associated molecules *GAP43* and *SCG-10*, the cell-recognition molecules *L1* and *CHL1* as well as transcription factor *c-Jun* and *ATF3* mRNA levels remain unaffected when a CST lesion is inflicted at the level of C3/C4 (Mason *et al.*, 2003). In contrast, they are all up-regulated when the injury is inflicted within layer V of the neocortex and at a maximum distance of 300 to 500 μm from the neuronal cell bodies (Mason *et al.*, 2003). In either case, *CAP-23* and *krox-24* mRNA levels appear unaltered. The above mentioned molecules are thought to be involved in the response mounted by neurons following an injury (Herdegen *et al.*, 1993a; Herdegen *et al.*, 1993b; Benowitz and Routtenberg, 1997; Herdegen *et al.*, 1997; Takeda *et al.*, 2000; Mason *et al.*, 2000; Zhang *et al.*, 2000; Tsujino *et al.*, 2000; Chaisuksunt *et al.*, 2000a; Chaisuksunt *et al.*, 2000b; Mason *et al.*, 2002; Nakagomi *et al.*, 2003; Campbell *et al.*, 2005).

The fact that *GAP-43* is not up-regulated in response to cervical level dorsal column injury confirms earlier reports regarding its patterns of expression in the injured CST (Anderson *et al.*, 1998; Vaudano *et al.*, 1998). Also, a CST lesion exacted within the posterior limb of the internal capsule leads to a 2.5 fold decrease in GAP-43 IR at six weeks p.i (Vizzard, 1999). Notably, Mason *et al.* (2003) report that GAP-43, SCG-10, L1, CHL1, ATF3 and c-Jun mRNA are present in CST neurons in uninjured animals and in relatively high amounts. This, according to the authors, might explain the ability of CST fibres, axotomised at the spinal cord level, to produce local sprouts (Bregman *et al.*, 1989; Schnell *et al.*, 1994), despite the lack of any enhanced expression of growth-promoting

molecules. In a recent study it was possible to enhance the regenerative response of axotomised CST neurons by causing inflammation around neuronal somata by means of a single lipopolysaccharide (LPS) application to the motor cortex. This resulted in the upregulation of c-Jun, ATF3, SCG-10 and GAP-43 expression in CST neurons which was maintained for two weeks following LPS administration (Hossain-Ibrahim *et al.*, 2006). Moreover, the degree of inflammation appeared to correlate to the level of gene expression achieved. Despite this, the authors report a failure of CST neurons to mount a significant regenerative response and attribute this to the inability of this method to sustain a widespread and long-term expression of these growth-associated molecules.

4.1.4 Axonal regeneration approaches in CST regeneration

A lot of technically varied approaches have been utilised to bring about a potentially regenerative outcome on the injured spinal cord (Chapter 1 and for reviews: Hunt *et al.* 2003; Schwab 2004; Ramer *et al.* 2005; Teng and Tang 2005). Targeting of myelin-associated inhibitors, cellular transplantation and neurotrophin delivery, have all been part of such attempts. Here, reports of the use of these techniques as they are applied to CST regenerative studies are summarised.

4.1.4.1 Counteracting myelin inhibitors in injured CST axons

The use of IN-1, the anti-Nogo A monoclonal antibody (Caroni and Schwab, 1988a; Caroni and Schwab, 1988b) against the exon 3 encoded N'-terminal domain of Nogo-A (Fiedler *et al.*, 2002), has been reported to have some promise at promoting regeneration in the rat CST injury model even though it has been less successful in other CNS tracts such as injured retinal ganglion neurons (Cui *et al.*, 2004) and ascending dorsal columns (Oudega *et al.*, 2000). Adult rats treated with IN-1, delivered by means of a hybridoma cell line, show enhanced sprouting with some axons apparently regenerating up to 11 mm caudal to the lesion (Schnell and Schwab, 1990) in treated animals, as opposed to 2.6 mm in control animals. However, most subsequent studies show no regeneration at all in the untreated CST. The effects of IN-1 infusion on CST regeneration was later

shown to be enhanced when it was used in conjunction with foetal spinal cord grafts (Schnell and Schwab, 1993). This effect was further amplified with the co-administration of NT3 (Schnell *et al.*, 1994), with axotomised CST axons apparently regenerating up to 20 mm distal to the lesion site. In addition to its effects on CST regeneration, IN-1 administration also affects sprouting of uninjured as well as axotomised fibres. IN-1 infusion induces ipsilateral but aberrant CST sprouting rostral to a unilateral pyramidotomy and contralateral sprouting caudal to a pyramidotomy (Bareyre *et al.*, 2002). This aberrant and ectopic growth of regenerating CST fibres following a pyramidotomy and subsequent IN-1 treatment, has been noted in an earlier study in which regenerating axons were found in the dorsal and lateral columns but did not follow the path of the uninjured CST fibres (Raineteau *et al.*, 1999). Interestingly, IN-1 administration alone has been shown to lead to the up-regulation of a variety of growth-associated genes (GAP43, actin, myosin), growth factors (BDNF, vEGF) and transcription factors (STAT) in rat spinal cords. Administration of IN-1 following a pyramidotomy leads to the additional up-regulation of growth factors (Bone Morphogenic Proteins), growth factor receptors (GDNF receptor) and important axon guidance cues (semaphorins 3 and 6) (Bareyre *et al.*, 2002).

Even though the study by Bareyre *et al.* (2002) does not provide a link between the observed up-regulation and regenerating CST fibres, it may be hinting to the fact that reports of functional recovery may result from the ability of IN-1 to enhance CST plasticity by counteracting its targets, thus favouring the creation of a more regeneration-friendly environment that can support re-growth around partial or incomplete lesions (Raineteau *et al.*, 2002). This would be in line with the observation that CST cell bodies normally contain very high levels of *ngr*, *nogo-66* and *nogo-a* mRNAs (Hunt *et al.*, 2002b; Hunt *et al.*, 2003), while CST fibres around the lesion site strongly express the Nogo-A protein (Hunt *et al.*, 2003). The application of IN-1 has now been extended to non-human primates, with regeneration being shown in marmosets that have previously received a unilateral CST lesion at the thoracic level. mAb IN-1 treated animals show sprouting and regeneration of injured CST fibres with a few fine axons growing through and up to 5 mm caudal to the lesion site (Fouad *et al.*, 2004).

Generally, the use of the mAb IN-1 is not without its pitfalls since Nogo-A may not be its sole antigen. The exact nature of the molecules neutralised by the mAb IN-1 has not been thoroughly characterised. In order to overcome this drawback highly purified, monoclonal, mono-specific, IgG antibodies to Nogo-A have been produced: 11C7, raised against the rat sequence of amino acids 623 to 640 and 7B12 targeting amino acids 1 to 979 (i.e. the C-terminal part of the Nogo-A specific region) (Oertle *et al.*, 2003). Their use *in vivo* has recently been documented in the CST regeneration model. Two week long intrathecal infusion of 11C7 and 7B12 antibodies immediately following a CST dorsal column lesion, reportedly leads to enhanced CST regeneration with fibres following an aberrant route and growing around 2 to 5 mm caudal to the lesion site (Liebscher *et al.*, 2005). Some recovery of function was also reported but as in most studies of this type the effect that IN-1 has on local plasticity should be taken into account. Reports of success with antibodies against Nogo have been criticised with regards to the origin of regenerating fibres, as most studies employ partial dorsal column lesions (Hunt *et al.*, 2002a; Schwab, 2004; Teng and Tang, 2005). This popular model unfortunately allows for the possibility that the apparently regenerating CST axons may originate from the intact ventral CST component which has been demonstrated to confer considerable plasticity to the injured CST if left unscathed (Weidner *et al.*, 2001; Raineteau *et al.*, 2001).

In addition to the use of IN-1, the Nogo extracellular peptide residues 1 to 40 (NEP1-40 peptide), which competes with Nogo-66 for binding to the NgR, has also been shown to have an effect on CST regeneration. Intrathecal infusion of the antagonistic peptide via an osmotic mini-pump in adult rats with a mid-thoracic spinal cord dorsal hemisection resulted in a ≈10 fold increase of CST axonal sprouting. This regeneration was accompanied by some degree of functional recovery (GrandPre *et al.*, 2002). Interestingly, the CST regenerating fibres appeared to follow a random route through the white and grey matter of the spinal cord, perhaps reflecting their growth through a debris-filled substrate. Subcutaneous administration of NEP1-40 results in the extensive growth of corticospinal axons, up-regulation of axonal growth promoting protein small proline-rich repeat protein 1A (SPRRP1A) (Bonilla *et al.*, 2002) and synapse re-

formation following a thoracic level lesion in mice, an effect not abolished by a 1 week delay between lesioning and NEP1-40 treatment (Li and Strittmatter, 2003). It has been suggested that NEP1-40 may block inhibitory responses to Nogo as well as to MAG and OMgp (Schwab, 2004). Intrathecal administration of NgR(310)ecto-Fc antagonist, the Fc bound, soluble, truncated form of the NgR (Fournier *et al.*, 2002), following a mid-thoracic dorsal over-hemisection promotes significant CST and raphaespinal axonal sprouting which is accompanied by the recovery of electrophysiological function (Li *et al.*, 2004a). A new anti-NgR monoclonal antibody capable of inhibiting the binding of Nogo, MAG and OMgp has now been discovered and has been shown to block myelin inhibition at least on early post natal DRG neurons *in vitro* (Li *et al.*, 2004b). No reports of its effect *in vivo* exist as yet.

Other attempts at counteracting myelin inhibitors for CST regeneration target the second messengers activated downstream of NgR. Ligand-bound NgR associates with LINGO-1 and p75 or TROY to activate the RhoA GTPase. Rho has been shown to be activated by Nogo-66 binding to NgR *in vitro* (Fournier *et al.*, 2003). The active, GTP-bound RhoA further activates ROCK, which in turn transduces the inhibitory effect of myelin to the neuronal cytoskeleton. This pathway has been targeted for the induction of CST regeneration using either C3-ADP-ribosyltransferase (C3 transferase) to inhibit RhoA (Aktories *et al.*, 1989; Lehmann *et al.*, 1999) or the pyridine derivative Y-27632 which targets ROCK. When C3 transferase, the RhoA specific inhibitor was delivered into the thoracic adult mouse spinal cord using a fibrin adhesive gel, extensive long distance CST regeneration and up-regulation of *gap-43* mRNA levels in neurons within the motor cortex was observed (Dergham *et al.*, 2002). The authors themselves acknowledge that they cannot discount the possibility that this was due to the enhanced plasticity of spared fibres since this was a dorsal over-hemisection lesion. Different results were obtained when C3 was used in a dorsal hemisection model in the adult rat but was instead applied utilising a mini-pump to gradually release it over a three-week period. In this case, C3 appeared unable to promote or sustain any long term CST regeneration but had a beneficial effect in reducing scar formation (Fournier *et al.*, 2003). The authors suggest that this discrepancy

between the two administration methods was due to the inability of C3 to gain access to the axotomised CST fibres. This suggestion is a valid possibility considering that the C3 24 kDa protein does not readily penetrate the cell membrane and may therefore be needed in high amounts in order to induce an effect. It would be interesting to see the effects of using the more permeable C3 chimeric protein analogues, such as the C3-05 analogue, in terms of CST regeneration (Winton *et al.*, 2002). Equally plausible is that the acute administration of a massive 50 μg dose of C3 immediately after lesioning had a greater neuroprotective effect than that possibly achieved with the slow infusion of 0.75 μg/hr over three weeks (Dergham *et al.*, 2002; Fournier *et al.*, 2003) and increased the number of spared fibres. This would also make sense since administering a C3-05 Rho antagonist within the transected spinal cord increases neuroprotection, with a 50% reduction of TUNEL labelled cells in mice or rats, perhaps by inhibiting the expression of $p75^{NTR}$ (Dubreuil *et al.*, 2003). Unfortunately, in the same study no data were presented in relation to any regeneration of CST or other fibres. It has been shown that the NgR can activate Rho in a $p75^{NTR}$ dependent manner (Wang *et al.*, 2002a; Yamashita and Tohyama, 2003). The role played by $p75^{NTR}$ in transducing an inhibitory signal via NgR is by no means clear as yet since contradictory reports exist as to its function. Lack of $p75^{NTR}$ expression in transgenic mice does not appear to promote a CST regenerative response (Song *et al.*, 2004; Zheng *et al.*, 2005). A recent *in vitro* study using siRNA technology to knock down the expression of $p75^{NTR}$, NgR or RhoA demonstrated that reducing RhoA expression enhances the ability of DRG axons to grow in a myelin-extract substrate, more prominently than sole $p75^{NTR}$ or NgR silencing (Ahmed *et al.*, 2005). It is worth pointing out that only Western blots were shown as proof of reduction in the levels of either of the proteins targeted. It would have been interesting to see a reduction in actual mRNA levels by northern blot analysis or rt-PCR since this would be a more sensitive detection method. Ahmed *et al.* (2005) go on to suggest that knocking down $p75^{NTR}$ and NgR does not block those inhibitory signals not derived by myelin, such as those derived by CSPG, semaphorins or ephrins, that also converge on RhoA and have been shown to cause growth cone collapse (Hunt *et al.*, 2002a; Sandvig *et al.*, 2004; Ahmed *et al.*, 2005).

Inactivation of ROCK via Y-27632 (Hirose *et al.*, 1998), induced comparable CST regeneration despite the difference in species and administration methods. However, C3 appears to make a bigger impact on CST regeneration that Y-27632 which could suggest that RhoA may be a more influential target than ROCK for CST regeneration studies. In another study, the protein kinase C Alpha inhibitor Gö6976 was used to block the activation of Rho via myelin components. When Gö6976 was administered intrathecally following a dorsal hemisection it lead to the regeneration of dorsal column axons but not CST fibres (Sivasankaran *et al.*, 2004). Myelin associated growth inhibitors can be counteracted by immunisation with myelin (Huang *et al.*, 1999). Adult mice immunized in this manner showed extensive regeneration of large numbers of CST axons after a dorsal hemisection of the spinal cord. This anatomical regeneration led to recovery of certain hind limb motor functions. Again, to which degree the functional regeneration observed was solely due to CST regeneration cannot be determined. Immunisation with recombinant Nogo-66 (Wang *et al.*, 2002b) and rMAG (McKerracher *et al.*, 1994) of adult mice that had previously received a dorsal hemisection resulted in sprouting and long distance regeneration of CST fibres (Sicotte *et al.*, 2003). In animals that were immunised with the rNogo-66/rMAG, CST axons reached ≈8.4 mm caudal to the lesion. The authors of this study acknowledge that the ventral CST component also sprouts in response to the rNogo-66/rMAG treatment. In another approach aiming to narrow the extensive range of molecules targeted by the myelin vaccine, a recombinant plasmid DNA vaccine was utilised (Xu *et al.*, 2004). The vector was designed to express a fusion protein composed of 4 domains: the Ig 1-5 domain of rat MAG (McKerracher *et al.*, 1994), the EGF-L domain of human TN-R (Xiao *et al.*, 1996) as well as the N-terminal and Nogo-66 domains of human Nogo-A (GrandPre *et al.*, 2000) under the control of a CMV promoter (Xu *et al.*, 2004). This study, reports successful CST regeneration with fibres reaching 5 and in some cases 12 mm caudal to a dorsal column microlesion site. Some functional recovery was also reported. The success achieved with studies targeting the disruption of the effects of Nogo or NgR at the protein level is not mirrored with attempts at disrupting these two factors at the genetic level (for a review: Teng and Tang, 2005). Disruption of the *Nogo* gene by different groups produced

varied success in a dorsal hemisection paradigm ranging from significant CST regeneration in young adult mice lacking Nogo-A/B (Kim *et al.*, 2003) to moderate CST regeneration in Nogo-A knock-out mice (Simonen *et al.*, 2003) to no effect observed in either Nogo-A,B or Nogo-A,B,C mutants (Zheng *et al.*, 2003). The same variable pattern is present in the case of *NgR* transgenic animals. In *NgR* null mutants that received a dorsal hemisection, no CST axonal regeneration was evident (Kim *et al.*, 2004). However, there was a remarkable improvement in locomotor function, even in animals with a complete spinal cord lesion. This could be attributed to the successfully regenerating raphaespinal and RST fibres (Kim *et al.*, 2004). A similar study, using a different strain of *NgR* null mice also reported no CST regeneration following a dorsal hemisection at the thoracic level (Zheng *et al.*, 2005). It has been suggested that injured CST axons of NgR knock-out transgenic mice may be unable to mount a regenerative response, even if their environment favours it, due to their potential inability to up-regulate regeneration enhancing factors since they have never been exposed to an environment containing NgR in the first place (Teng and Tang, 2005). Studies in transgenic animals could also be viewed to reinforce the growing consensus that the interaction of Nogo-A and NgR is not a pure inhibitory one for axonal regeneration (Hunt *et al.*, 2002a; Hunt *et al.*, 2003; Sandvig *et al.*, 2004; Raisman, 2004; Teng and Tang, 2005). Rather than having a purely inhibitory function, these molecules could have more of a guidance role on myelinated tracts such as the CST as a means of ensuring correct target innervations. Abolishing them, may hinder any regenerative attempt these fibres may mount (Raisman, 2004; Teng and Tang, 2005). Several studies have addressed the effect of extra-cellular matrix molecules on CST regeneration. Chondroitin sulphate proteoglycans (CSPGs) are inhibitory to axonal growth, a function attributed to the CS side-chains since their inhibitory effect can be opposed by the chondroitinase ABC (ChABC) enzyme (Rhodes and Fawcett, 2004). When ChABC was infused intrathecally into rats that had received a C5/C6 dorsal column crush lesion, resulted in some regeneration of CST axons accompanied by some arborisation into the grey matter with up-regulation of GAP43 (Bradbury *et al.*, 2002).

4.1.4.2 Cellular transplantation and CST regeneration

Schwann cells (SCs), olfactory ensheathing glial cells (OEGCs), peripheral nerve (PN) and foetal spinal cord grafts as well as genetically modified non-neuronal cells have all been utilised to promote CST regeneration in either transection or contusion injury models.

Schwann Cells

When a peripheral nerve graft is transplanted into the cut stump of an axotomised optic nerve, regeneration of RGC axons occurs (Berry *et al.*, 1986; Vidal-Sanz *et al.*, 1987). This effect was attributed to the presence of SCs surrounding the sciatic nerve axons (Hall and Berry, 1989). SCs have been shown to be capable of expressing BDNF (Meyer *et al.*, 1992), NGF (Assouline *et al.*, 1987; Tuszynski *et al.*, 1998) and CNTF (Friedman *et al.*, 1999). However, the regenerative ability of SCs has so far not been realised whenever they have been applied to CST regeneration paradigms. CST axons, along with Purkinje cells, show an inability to regenerate their axons into grafts that contain SCs (Richardson *et al.*, 1982). Studies that utilised SC-seeded guidance channels thus removing the possibility that other cells present in a PN, such as fibroblasts, could confound the experiment, confirmed the previous reports of no CST axonal regeneration into PN grafts (Houle, 1991; Xu *et al.*, 1999). This is true even when SC transplantation was accompanied by exogenous administration of neurotrophins such as NT3 (Xu *et al.*, 1995) or when SCs were transduced by a retroviral vector to secrete NGF (Tuszynski *et al.*, 1998), BDNF (Menei *et al.*, 1998) or NT3 (Blits *et al.*, 2003). However, it is worth pointing out that in a separate study when SC transplantation was accompanied by intrathecal infusion of NT3 or/and BDNF, regenerating axons were noted up to 6 mm into the distal stump (Bamber *et al.*, 2001). In this case, neurotrophic factors were infused at significantly higher amounts than the more biologically reasonable amounts in the case of virally transduced cells (Tuszynski *et al.*, 1998). This allows for the possibility that cells other than neurons are involved in this response. Indeed astrocytes and oligodendrocytes also express the truncated versions of the trkB and trkC receptor mRNAs (Condorelli *et al.*, 1995; Wang *et al.*, 1998; Bamber *et al.*, 2001). Recent studies suggest that the classical view that the truncated forms of

the trk receptors are detrimental to regeneration may not be the whole story. For example, it has been shown that truncated TrkB receptors can mediate the endocytosis of extracellular BDNF and storage into an intracellular pool (Alderson *et al.*, 2000). It therefore follows that despite their lack of tyrosine kinase activity truncated, Trk receptors may still be capable of some intracellular signalling. This is supported by the recent demonstration that the binding of BDNF to its truncated receptor on astrocytes activated the release of Ca^{2+} from intracellular stores through a signalling pathway that involves an as yet unidentified G protein *in vitro* (Rose *et al.*, 2003; Reichardt, 2003). Inducing SC grafts to over-express BDNF and NT3 by means of an adenoviral vector failed to induce CST axons to enter the graft and grow into the distal stump following a thoracic level lesion (Blits *et al.*, 2003). These results were contradicted when in a recent study, genetically modified NGF/BDNF over-expressing SC grafts were administered in combination with foetal spinal cord cell suspension in a contusion CST lesion model. The authors report that some CST axons extend into and beyond the lesion site. The functional recovery reported however cannot be attributed solely to CST arborisation (Feng *et al.*, 2005). Again, differences in the lesioning models used in these two studies could account for this discrepancy and the study by Feng *et al.* (2005) would ideally need to be repeated.

Olfactory ensheathing cells

Like SCs, OEGCs have been shown to express neurotrophin factors and adhesion molecules (Barnett, 2004). Studies of purified OEGC cultures have detected mRNA for NT4/5, NGF, BDNF and neuregulin but not for CNTF or NT3 which would be beneficial for CST regeneration (Thompson *et al.*, 2000; Boruch *et al.*, 2001). Many contradictory reports have made it difficult to reach a conclusion as to the efficacy of OEGCs in regeneration studies. This may be related to differences in the lesion models employed, in the preparation of OEGC or differences in the mixture of cells in the OEGC population used. Some researchers suggest that OEGCs have the capacity to positively influence CST regeneration and avert secondary damage of the spinal cord by cavitation (Ramon-Cueto *et al.*, 2000; Nash *et al.*, 2002; Keyvan-Fouladi *et al.*, 2003). In a report of significant CST regeneration, a few fibres were able to grow up to 1.5

cm caudal to the lesion site following complete spinal cord transection. In this experiment purified OEGCs were administered at 1 mm either side of the centre of the lesion (Ramon-Cueto *et al.*, 2000). In these experiments, animals were able to recover some function. In another study by Keycan-Fouladi *et al.* (2003), animals received a unilateral focal electrolytic lesion in the medioventral, descending dorsal columns in the C1-C2 level. Transplantation of OEGCs was carried out eight weeks following the injury. Regenerating CST fibres were reportedly present for up to 10 mm caudal to the lesion/OEGC transplantation sites. Even though there is the possibility that this was due to spared fibres, the authors report restoration of paw reaching function to about 50% of normal. The same can be said for an earlier study utilising this lesioning model but without the delayed transplantation and carried out by the same laboratory (Li *et al.*, 1997). Transplantation of OEGCs has recently been shown to enhance collateral branching from spared ventral CST branch (Chuah *et al.*, 2004), suggesting that recovery of function after partial lesions may be brought about by factors other than axonal regeneration.

Other reports contradict the OEGC's regenerative effect with a distinct lack of any significant regenerative response. A contusion lesion of the descending CST that spares the lateral funiculi and subsequent OEGC transplantation failed to promote regeneration or dieback of injured descending CST axons. Some local sprouts attributed to local DRG or spinal interneurons appeared to follow an irregular route following the also random migration pattern on the transplanted OEGCs (Nieto-Sampedro, 2003). These authors suggest that the CST may be one of the tracts that cannot be repaired by the sole transplantation of OEGCs. The authors suggested that whether or not OEGCs can promote regeneration or not depends on their ability to migrate within the injured CNS tissue while their high susceptibility to the free-radicals present within the environment of a contusion injury limits their ability to do so (Nieto-Sampedro, 2003). In another study, comparing the efficacy of SCs and OEGCs transplanted within a contusion lesion at the thoracic level, demonstrated that OEGCs did have a beneficial effect on reducing dieback, cavitation and promoting re-myelination but did not lead to any axonal regeneration through the transplant (Takami *et al.*, 2002). This

discrepancy in achieved regeneration via the OECG transplantation approach is perhaps a reflection of the somewhat different lesioning models of spinal injury and transplantation paradigms utilised in the various studies. The less successful but more severe contusion lesion (Hill *et al.*, 2001) is particularly effective at destroying dorsal CST axons over a distance of several millimetres and produces massive cavitation in the rat spinal cord whereas the more elegant but ambiguous electrolytic lesion (Li and Raisman, 1995) produces minor scaring. However, even though it might be expected that combining the scar penetrating properties of OEGCs and the axonal growth promoting effect of Schwann cells would have a greater potential for inducing CST regeneration, a recent report disputed that effect. In a complete transection model of regeneration, Schwann cell grafts were combined with OEGCs and chondroitinase ABC (ChABC) administration. Unlike other supraspinal populations, CST axons still failed to transverse the scar (Fouad *et al.*, 2005). A number of experiments aimed at addressing the combinatorial approach of delivering OEGCs that have been transduced to express neurotrophic factors have recently been attempted. Using an adenovirus vector, OEGCs transduced to express NT3 were transplanted within the lesion site of a unilateral C4 transection encompassing the RST. The functional recovery reported may or may not be attributable to CST axonal regeneration (Ruitenberg *et al.*, 2005) and could be attributed to regenerating RST fibres.

Foetal cell transplants

Foetal tissue has been used in studies targeting the axotomised CST on the assumption that it can provide permissive conditions for regeneration. However, unaided, CST axons cease any regeneration at the host/transplant interface (Bregman *et al.*, 1997a). When E14 spinal cord transplants are accompanied by the simultaneous intrathecal administration of NT3, NT4/5, BDNF supraspinal fibres appear to have an increased growth capacity (Bregman *et al.*, 1997b). In this series of experiments, axons were able to enter and grow within the graft. This effect is amplified when the foetal transplant and neurotrophin treatment is delayed by 2-4 weeks after a complete spinal cord transection, suggesting that chronic injuries can still be addressed and intervention is still possible (Coumans *et al.*, 2001).

Engineered fibroblasts

Ex vivo gene therapy employing modified fibroblasts has been used in many experiments with varied success as far as CST regeneration is concerned. When fibroblasts engineered via a retroviral vector to express NGF, BDNF, NT3 or bFGF were transplanted into the grey matter of a previously uninjured spinal cord, it was mostly sensory axons of DRG origin rather than CST axons that were induced to enter the transplant (Nakahara *et al.*, 1996). A later study revealed that sensory axons are particularly attracted to the secreted NGF and they grow towards it in a directionally sensitive manner, while at the same time CST axons are not attracted by this neurotrophin (Giehl and Tetzlaff, 1996; Tuszynski *et al.*, 1997; Grill *et al.*, 1997b). However, NT3 secreting fibroblasts able to support transgene expression for around three months, were able to stimulate CST axonal elongation following a dorsal column over-hemisection (encompassing the rubrospinal and coeruleospinal projections) (Grill *et al.*, 1997a). These authors report that in the NT3 grafted animals, CST axons extended for up to a maximum of 8 mm caudal to the lesion site, a distance corresponding to the boundary of NT3 diffusion from the graft itself but this growth was based on the host ventral grey matter acting as a permissive growth substrate and was not supported by the collagen containing fibroblasts graft itself (Grill *et al.*, 1997b). Unfortunately, even though this study does provide evidence that the transgene is expressed at the longest time point examined, the amount of NT3 protein produced was not measured (Grill *et al.*, 1997a). The same laboratory went on to establish that delivering BDNF via genetically engineered fibroblasts transplanted into a sub-cortical lesion, does promote the survival of ≈89% of axotomised CST axons compared to only ≈36% in untreated animals (Lu *et al.*, 2001). The same grafts however failed to promote any CST axonal elongation when implanted either sub-cortically or within a thoracic dorsal column lesion.

Delaying grafting of NT3 secreting fibroblasts for three months following a dorsal column thoracic hemisection was reportedly still within the window of opportunity for inducing a regenerative effect in injured CST axons via this approach (Tuszynski *et al.*, 2003). In this study, the authors report that following a three-month treatment with the transduced fibroblasts, CST axons were able to

regenerate for up to an impressive 15 mm distal to the lesion site. Within the same year however a similar study published by a different group, report opposing results. Delaying the grafting of BDNF and NT3 secreting modified fibroblasts into a six week chronic CST cervical hemisection results in limited regeneration even though enhanced neuroprotection (attributed to BDNF) is reported (Shumsky *et al.*, 2003). This is despite the use of both cyclosporin and methylprednisolone that may have contributed to the neuroprotection observed (Oudega *et al.*, 1999; Diaz-Ruiz *et al.*, 2000). The first study by Tuszynski *et al.* (2003) employed an incomplete lesion and therefore the possibility of the regeneration observed being from the ventral CST component cannot be discounted. At the same time however, the second study by Shumsky and colleagues (2003) fails to report the exact number of genetically engineered fibroblasts transplanted into the lesion site and it is, in this respect, difficult to compare the effectiveness of their approach to that of other laboratories employing this technique. Another difference between these two studies is that the Tuszynski group delivered their genetically engineered cells by means of an amorphous and relatively liquid preparation while the second group utilise foam gel soaked in the cells. The fact that these two materials have different physical properties would potentially have an impact the diffusion rate of the secreted NT3 as well the viability of the transplanted cells. Over-expression of the cytokine leukaemia inhibitory factor (LIF), a modulator of neuronal and glial function during development, by means of transduced fibroblasts also leads to a growth promoting effect on axotomised CST axons (Blesch *et al.*, 1999). When the modified fibroblasts are transplanted within the lesion site of adult rats, LIF appears to promote a selective increase of NT3 secretion at the lesion site. This is accompanied by modest CST regeneration through the grey matter of the host tissue. Interestingly, LIF over-expression has no effect on BDNF, CNTF, GDNF or NGF expression at the lesion site (Blesch *et al.*, 1999).

Peripheral nerve grafts

Peripheral nerve implantation (Houle, 1991) especially when accompanied by neurotrophin administration, has also been shown to induce a CST regenerative response. Administration of αFGF mixed in with fibrin glue and transplanted

along with 18 intercostal nerve pieces into a CST complete transection site, induced some regeneration into and through the graft (Cheng *et al.*, 1996). Similarly when intercostal nerves were transduced by means of an adenoviral vector to secrete NT3 transiently and then transplanted into a dorsal hemisection site, CST axons regenerated for up to 8 mm distal to the lesion site but using the host grey matter as a medium and not the graft itself (Blits *et al.*, 2000). A few years later, the same laboratory utilised adeno-associated vectors expressing BDNF and NT3 to transduce intercostal nerves and then transplanted them into a complete thoracic spinal cord transection site. The authors report that CST axons were stimulated to sprout but none were seen to enter the tissue up to 16 weeks following transplantation and vector administration (Blits *et al.*, 2003).

4.1.5 Neurotrophins and CST regeneration

The effectiveness of different neurotrophic factors in promoting a regenerative effect in axotomised neurons of the CST has been addressed in many and varied experimental paradigms. In this study emphasis is placed on NT3 since, as explained in the following sections, it can promote a robust and consistent outgrowth of CST axons.

4.1.5.1 Neurotrophin 3

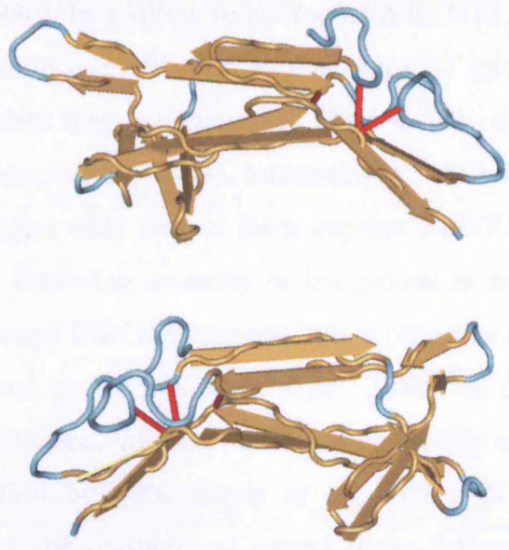
NT3 is a cysteine-knot neuronal growth factor (McDonald and Hendrickson, 1993). The NT3 cDNA encodes a 258 amino acid long pre-pro-neurotrophin with a signal peptide and a pro-protein (pro-NT3), both of which are proteolytically processed to generate the ≈119 amino acid long mature protein (Hohn *et al.*, 1990; Maisonpierre *et al.*, 1990b). The overall secondary structure of NT3 (Figure 4.1.5-a, panel **A**) is similar to that of other neurotrophins (Hohn *et al.*, 1990), with each protomer forming a four-stranded β-sheet and a small fraction of α-helix with three disulfide bonds (Butte *et al.*, 1998). X-ray crystallography studies have shown NT3 to be a non-covalently linked homodimer (McDonald and Hendrickson, 1993), whose formation is essential for NT3-specific TrkC receptor activation (Butte *et al.*, 1998). Residue Arg103 of the NT3 protein is

important for binding affinity to the TrkC receptor (Figure 4.1.5-a, panel **B**) while specificity is attributed to residue Gln83 (Butte *et al.*, 1998). Both the mature and the pro-neurotrophin forms are thought to be capable of eliciting a biological effect. The pro-form appears to bind preferentially to the $p75^{NTR}$ receptor inducing apoptosis, while the mature form has a higher affinity for the TrkC receptor and promotes survival (Lee *et al.*, 2001). The intracellular processing, sorting and concomitant secretion routes of pro-NT3 have been the focus of debate with somewhat contradictory reports. Some researchers suggest that processing and sorting of pro-NT3 occurs primarily via the constitutive secretory pathway, where mature NT3 is released following the highly efficient, furin-mediated, pro-NT3 endoproteolytic cleavage in the *trans*-Golgi network (Mowla *et al.*, 1999; Farhadi *et al.*, 2000). Recent studies suggest that cells may be capable of shunting the processing of pro-NT3 to the regulated secretory pathway, which results in mature NT3 being stored in secretory granules. Over-expressing of pro-NT3 by means of transient transfection methods can lead to such rerouting. This re-routing is consistent with the dual role of NT3. As a neuronal survival factor (Kalcheim *et al.*, 1992) it requires constant release via the constitutive secretory pathway while as a modulator of synaptic plasticity (Kang and Schuman, 1995) and depolarisation (Kafitz *et al.*, 1999) it is released in response to extracellular stimuli via the regulated secretory pathway instead (Farhadi *et al.*, 2000). NT3 is a target derived neurotrophic factor with survival promoting, differentiation and plasticity modulating functions. A deficiency in NT3 expression, as elucidated in mutant mice lacking NT3, results in an abnormal proprioception as cutaneous mechanoreceptors are lost during development while spinal afferents, muscle spindles and Golgi tendon organs never form (Airaksinen *et al.*, 1996). In addition, most classes of DRG neurons are severely reduced in numbers but are not totally absent (Airaksinen and Meyer, 1996). Other laboratories, employing different techniques for NT3 gene ablation have to come to similar conclusions, reporting severe movement deficits (Ernfors *et al.*, 1994), an overall failure to thrive and early death (Tessarollo *et al.*, 1994). It was recently demonstrated that the ability of NT3 to promote axonal elongation may be due to the fact that it can inhibit the activation of glycogen synthase kinase-3β (GSK3β) which normally deactivates the collapsing response

mediator protein-2 (CRMP-2) (Yoshimura *et al.*, 2005). CRMP-2 is involved in microtubule reorganisation and therefore axonal elongation and branching (Fukata *et al.*, 2002) and the fact that NT3 can prolong its active state can be linked to its regenerative potential. Neurons of the CST express TrkC and TrkB receptors (Giehl and Tetzlaff, 1996; Giehl, 2001). In addition, TrkC protein and mRNA levels remain constant throughout human development and they are present in glial, pyramidal and non-pyramidal cells in the motor cortex (Beltaifa *et al.*, 2005). Research in rodents has shown that *trkC* mRNA levels are not down-regulated in injured CST neurons examined following a T8 contusion injury model (Liebl *et al.*, 2001). It therefore follows that NT3 administration would be expected to have a positive impact on CST regeneration.

The regenerative potential of NT3 was demonstrated early on in spinal cord regenerative studies following protein administration at the lesion site either acutely by means of a mini-pump (Schnell *et al.*, 1994; Bradbury *et al.*, 1999) or by slow release via a collagen implant (Houweling *et al.*, 1998) or via viral vector mediated delivery (Dijkhuizen, 1999; Blits *et al.*, 2003). Sprouting and axonal elongation was noted but no CST fibres were able to exit the scar tissue and grow distally. Even though the full-length TrkC and TrkB receptor levels do not seem to be affected by axotomy, expression of their truncated and inactive forms is apparently upregulated near the lesion site (Frisen *et al.*, 1993a; Frisen *et al.*, 1993b; King *et al.*, 2000). Thus, larger amounts of NT3 would be necessary to induce a trophic effect in these neurons since the truncated receptors would mop up the neurotrophin but since they lack a catalytic domain they would be unable to convey their message. This is thought to be one of the reasons why sole administration of neurotrophic factors may not be enough to promote any meaningful CST regeneration. CST neurons exhibit marked atrophy (Giehl and Tetzlaff, 1996) and progressive axonal die-back, initiated as soon as 24 hours after injury (Sayer *et al.*, 2002). CST axonal die-back is relatively fast and it has been approximated to be 40 – 60 μm/day (Pallini *et al.*, 1988).

A. Neurotrophin 3



B. TrkC receptor

Figure 4.1.5-a: Predicted structures of NT3 & the TrkC receptor-binding domain.

Three dimensional representations of the predicted structures of **A:** the A chain of human NT3 (GI:4505469, MMDB10558) and **B:** the 5th domain (d5) of the human TrkC receptor (GI:5822441, MMDB11000) (Ultsch *et al.*, 1999) which is the binding site for human NT3 (Butte *et al.*, 1998). Structures were obtained using the Cn3D4.1 software, available at the NCBI website (http://www.ncbi.nlm.nih.gov). (Disulfide bridges are shown in red).

One week after an internal capsule lesion, 49% of CST neurons have atrophied (Giehl and Tetzlaff, 1996). In addition to its trophic role, NT3 has been shown to exert a modest neuroprotective effect on axotomised CST axons (Namiki *et al.*, 2000) but primarily when they are transected close to the cell body (Giehl and Tetzlaff, 1996; Hammond *et al.*, 1999). Interestingly, while most CST neurons express the TrkB receptor only few of them express BDNF. These expression patterns don't change following axotomy or exogenous neurotrophin treatment suggesting that axotomised CSN are supported via a paracrine BDNF-mechanism which can be stimulated by NT3 (Schutte *et al.*, 2000) via its interaction with TrkB. A seven day intrathecal infusion of NT3 is reportedly capable of reducing CST end-bulb formation by 60% (Sayer *et al.*, 2002). NT3 has also been beneficial in promoting the plasticity of spared fibres following CST axotomy and thus indirectly contributing to the restoration of function following a lesion. As already stated previously, spared CST axons will sprout into the denervated grey matter when NT3 is over-expressed by means of an adenoviral vector (Zhou *et al.*, 2003). However, a recent report appeared to suggest that NT3 may in fact inhibit collateral sprouting of spared CST fibres originating from within the denervated side (Hagg *et al.*, 2005). Hagg *et al.* (2005) went on to suggest that this unexpected effect may be a consequence of the upregulation of $p75^{NTR}$, observed in axotomised CST neurons one week following either an internal capsule lesion (Giehl *et al.*, 2001) or in the epicentre of a contusion injury (Reynolds *et al.*, 1991).

Large diameter 1α primary afferent sensory neurons have been shown to regenerate following dorsal root rhizotomy and anastomosis in response to adenoviral vector mediated NT3 transduction of local populations of neuronal and non-neuronal cells (Zhang *et al.*, 1998). This is logical considering that they do express the TrkC protein (McMahon *et al.*, 1994). In this experiment a conditioning peripheral lesion was also employed in order to enhance the expression of growth promoting molecules (Chong *et al.*, 1999) and activate the transcription factor, signal transducer and activator of transcription 3 (STAT3) (Qiu *et al.*, 2005). However, most regenerating axons did not exit the dorsal root entry zone (DREZ) possibly due to the presence of tenascin-R and NG2

expressing cells which were found bordering the entrance to the CNS (Zhang *et al.*, 2001; Grimpe *et al.*, 2005). These findings were in later years repeated by another laboratory but this time using an intrathecal delivery system for administering NT3 (Ramer *et al.*, 2002). The efficacy of intrathecally delivering NT3 in a similar lesioning model appears to vary depending on the time that treatment is initiated (Ramer *et al.*, 2001). Ramer and colleagues (2001) talk about a "two tiered inhibition". The first tier, the astrogliotic barrier, takes place immediately after injury and its effects are susceptible to the intrathecal delivery of NT3. The second tier, the degenerative barrier, sets in a week after the lesion is inflicted and coincides with the onset of myelin breakdown. Initiating NT3 treatment at this point cannot overcome the inhibitory signals at the DREZ (Ramer *et al.*, 2001). Regeneration of ascending sensory, dorsal column fibres has been reported to occur following the *in situ* infusion via an osmotic mini-pump of large amounts of NT3 for a period of four weeks (Bradbury *et al.*, 1999). The regeneration and elongation reported was impressive, with large myelinated αβ regenerating axons just falling short of reaching their targets in the gracile and cuneate nuclei. These findings however, have not been replicated by another research group.

In addition to its effects on neurons, NT3 has an effect on glial cells. NT3 has been shown to be mitogenic for oligodendrocyte progenitors *in vitro* (Marmur *et al.*, 1998). The *in vivo* role of NT3 with regards to activated microglia is not fully understood yet. These cells have the potential to express NT3 and TrkC themselves and they proliferate in response to exogenous NT3 administration (Elkabes *et al.*, 1996). This suggests that this neurotrophin has the potential to act both as a paracrine and/or autocrine factor (Nakajima and Kohsaka, 1998). One function of NT3 in these cells is to suppress the activation of the inducible form of nitric oxide synthase (iNOS) (Tzeng and Huang, 2003). This effect was elucidated in *in vitro* experiments and is as yet to be replicated *in vivo* but if true it would imply that NT3 can exert an anti-inflammatory effect that may be beneficial in the injured spinal cord environment.

4.1.5.2 BDNF

BDNF appears to confer neuroprotection on axotomised CST neurons more efficiently than NT3, but it is significantly less efficient at promoting regeneration. This holds true when this neurotrophin is injected directly into the spinal cord (Schnell *et al.*, 1994). Primary fibroblasts, transduced by a retroviral vector to express BDNF, grafted into a dorsal hemisection lesion site, failed to attract axotomised CST fibres (Nakahara *et al.*, 1996). Infusion of BDNF (12 μg/day for 7 days) was able to prevent the onset of atrophy on axotomised CST neurons (Giehl and Tetzlaff, 1996) while a piece of gel foam soaked with BDNF enhanced the survival of axotomised CST neurons but failed to promote any axonal regeneration (Ye and Houle, 1997). Intracortical infusions of BDNF, initiated one week after CST axotomy and carried out for a following two weeks, promoted CST survival for a further 42 days after the injury was inflicted (Hammond *et al.*, 1999). The same method however failed to promote any regeneration, even in the presence of a peripheral nerve graft (Hiebert *et al.*, 2002). In a recent study, rat spinal cords lesioned by a contusion injury had a combination of foetal spinal cord grafts and genetically modified Schwann cells over-expressing NGF and BDNF transplanted within the lesion site (Feng *et al.*, 2005). This combinatorial approach is capable of inducing some CST fibres to grow into and through the lesion site. BDNF is thought to exert these effects via a paracrine mechanism (Schutte *et al.*, 2000).

4.1.6 Rationale

This chapter describes the experiments employed to test the hypothesis that if vector HSV1.pR19CMVrNT3 is capable of transducing injured CST neurons to produce biologically active protein, then administration of this construct would lead to an increased regenerative response when used alone and augment the response mounted by the injured CST neurons in the presence of E14 spinal cord segments. This would suggest that the vector-mediated levels of NT3 positively influence the limited natural capacity of CST neurons to regenerate. For the purposes of testing this hypothesis vector HSV1.pR19CMVrNT3 was constructed and its ability to promote regeneration was evaluated either unaided, via sole

vector administration following a chronic lesion or in conjunction with an E14 spinal cord transplant. In addition, a new generation of minimally disabled HSV1 vectors were briefly examined for their efficacy in delivering transgenes to the CST via a dorsal column or cortical injection in the adult rat.

4.2 Methods

4.2.1 Vectors utilised in this Chapter

The following vectors were used in this study, either to induce regeneration or for the delivery of marker genes to the chronically injured CST of adult rats.

Virus	Abbreviation
1764/ICP27$^-$/ICP4$^-$/RL1$^-$/pR19CMVLacZ	HSV1.pR19CMVLacZ
1764/ICP27$^-$/ICP4$^-$/RL1$^-$/pR19CMVGFP	HSV1.pR19CMVGFP
1764/ICP27$^-$/ICP4$^-$/RL1$^-$/pR19CMVratNT3	HSV1.pR19CMVrNT3
1764/ICP27$^+$/ICP4$^-$/RL1$^+$/pR19EF1α GFP^{WPRE}	HSV1.pR19EF1α GFP^{WPRE}
1764/ICP27$^+$/ICP4$^-$/RL1$^+$/pR19EF1α LacZ^{WPRE}	HSV1.pR19EF1α LacZ^{WPRE}

Table 4.2.1-a: Vectors used in CST regenerative studies.

The construction and characterisation of vectors HSV1.pR19CMVGFP and HSV1.pR19CMVLacZ has previously been published (Lilley *et al.*, 2001b). Vectors HSV1.pR19EF1aGFP^{WPRE} and HSV1.pR19EF1aLacZ^{WPRE} were constructed by Dr J.A. Palmer.

4.2.2 Construction and characterisation of HSV1.pR19CMVr/NT3

As discussed in Chapter 3, the vector backbone that allows efficient transgene delivery to both injured and chronically injured spinal cord was 1764/ICP27$^-$ /ICP4$^-$/pR19CMV (HSV1.pR19CMV). In order to create a vector capable of delivering biologically active rat NT3 (rNT3) protein, it was first necessary to sub-clone the cDNA into the pR19CMV expression cassette, substituting the LacZ marker gene. This cassette is flanked by homologous HSV1 LAT sequences enabling it to be subsequently inserted into the viral genome by homologous recombination. Production of rNT3 and confirmation of its bioactivity was then confirmed *in vitro* and *in vivo*.

4.2.2.1 Construction of plasmid pGEM5/pR19CMVrNT3

cDNA encoding for rNT3 (GeneBank accession number M33968) was originally cloned as a *Xho*I PCR generated fragment into pBluescript® SK (Maisonpierre *et al.*, 1990a). rNT3 cDNA (800bp) was removed by digesting first with *Kpn*I, blunted using T4 DNA Polymerase and finally cut with *Hind*III. The fragment was then sub cloned into the pR19 shuttle plasmid flanked by LAT sequences, allowing insertion into the LAT region (Lilley *et al.*, 2001b). The reporter gene GFP of the vector backbone pGEM5/pR19CMVGFP, was removed using *Xho*I followed by blunt ending with T4 DNA polymerase and digestion with *Hind*III. Multiple pGEM5/pR19CMVrNT3 clones were screened for the correct restriction profile following *XhoI* digestion. Correct cloning of the neurotrophin 3 (*Ntf3*) gene was confirmed by sequencing, using a CMV forward primer as listed in Chapter 2.

4.2.2.2 Production of HSV1.pR19CMVrNT3 vector

Purified HSV1.pR19CMVGFP (1764/ICP27$^-$/ICP4$^-$/pR19CMVGFP) viral DNA was co-transfected with pGEM5/pR19CMVrNT3 plasmid DNA. The construct was recombined into the endogenous LAT of the HSV1.pR19CMVGFP backbone between the two *BstXI* sites (*nt*120,220 and *nt*120,408) (Lilley *et al.*, 2001b). Recombinant plaques were detected by their white phenotype (no GFP expression), which is indicative of the correct insertion of the pR19CMVrNT3 expression cassette in both LAT regions of the HSV genome. Plaque purification was carried out until all plaques had the recombinant, white phenotype. The new vector was termed HSV1.pR19CMVrNT3 (1764/ICP27$^-$/ICP4$^-$/pR19CMVrNT3). Correct insertion of the rNT3 gene was confirmed by Southern blotting on recombinant plaques, each originating from separate recombination events. The recombinant vector was propagated by standard cell culture techniques on 27/12/M:4 cells and viral stocks were produced as outlined in Chapter 2.

4.2.2.3 Southern blotting on HSV1.pR19CMVrNT3 plaques

Each purified recombinant HSV1.pR19CMVrNT3 viral plaque was used to infect $1x10^6$ 27/12/M:4 cells in a 36 mm well tissue culture plate. Viral DNA from each plaque was extracted as outlined in Chapter 2, digested with *Xho*I and run on a 2% (w/v) agarose gel. HSV1.pR19CMVGFP viral DNA digested by *Hind*III/*Xho*I (releasing a 600bp fragment corresponding to GFP) was used as a negative control while a *Xho*I digested plasmid pBKS-rNT3 was used as a positive control. The pBSK-rNT3 plasmid was digested with *Xho*I and the rNT3 800bp fragment produced was purified and labelled with αP^{34}-dCTP to create an rNT3 specific probe. A detailed account of the procedure is given in Chapter 2.

4.2.2.4 Isolation & concentration of secreted rNT3 from cultured BHKs

$1x10^6$ BHK cells in a 36 mm well were infected at an MOI of 1. Two days later the infected monolayer was washed twice with 0.1M PB and 500 µl of DMEM were aliquoted in each well. The cells were subsequently returned to a 37°C/5%CO_2 incubator. At the desired time point, the DMEM was collected and centrifuged at 2,000 rpm for 5 minutes to remove any cells present. The supernatant was then transferred onto a 500 µl Microcon column with a 10kDa molecular weight cut-off (G-10000, Green). The column was then spun at 11,500g for 25 minutes at 4°C. The total volume of the concentrated sample was 20-25 µl, which represents the amount of rNT3 secreted from $1x10^6$ BHK cells infected with HSV1.pR19CMVrNT3. These were used for further testing such as Western blotting, ELISA or neurite outgrowth assays.

4.2.2.5 ELISA assay on vector secreted rNT3 *in vitro*

Supernatants from $1x10^6$ BHK cells previously infected with HSV1.pR19CMVrNT3 (n=6) or HSV1.pR19CMVGFP (n=2) at an MOI of 1 or were mock infected (n=2). Samples were obtained as outlined in the previous section with the exception that supernatants were not concentrated. Instead, they were serially diluted from 1:2 down to 1:10. Samples were analysed using the NT3 E_{max}® Immunoassay system (Promega) according to manufacturer's instructions.

4.2.2.6 Western blotting

Secreted rNT3 from $1x10^6$ BHK cells previously infected with HSV1.pR19CMVrNT3 at an MOI of 1 was obtained as outlined above. Expression was analysed at 2, 4 and 8-hour time points. An equal volume of protein sample buffer [5% (v/v) b-mercaptoethanol, 50mM Tris-HCl pH 8.0, 2% (w/v) SDS, 6% (v/v) glycerol and 0.005% (w/v) bromophenol blue] was added to each sample. Samples were placed on ice immediately and subsequently denatured by heating to 95°C for 4 min. They were then cooled on ice for 2 minutes and either loaded onto a 15% SDS-polyacrylamide gel or stored at -20°C. SDS-PAGE, transfer and antibody incubations were performed as described in Chapter 2. The primary antibody used was obtained from rabbit polyclonal anti-rat NT3 (1:200) (N-20, Santa Cruz Biotechnology, Inc., SC-547). Equal protein loading was confirmed by evaluating band intensity on a duplicate coomassie stained gel.

4.2.2.7 Neurite outgrowth assay on E14 rat DRG explants

Lumbar E14 rat DRGs were isolated, embedded in collagen and maintained in a chemically defined medium (DMEM/F12 (3:1) supplemented with 200 μg/ml transferrin, 200 μM putrescine, 40 nM progesterone, 60 nM selenium, 10 μg/ml insulin 0.01% (w/v) albumin) for 24 hrs. The supernatant from $1x10^6$ BHK cells previously infected at an MOI of 1 with HSV1.pR19CMVrNT3 (n=3) or HSV1.pR19CMVGFP (n=3) (negative control) or mock-infected (n=2) was collected, clarified to remove cellular debris and concentrated as described in Chapter 2. The concentrated conditioned media was added to each collagen embedded DRG explant. This was repeated every day and neurite extension was compared 3 days later by immunofluorescent staining of the resulting neurites using mouse TUJ1 anti-neuron specific type III β tubulin antibody (1:5000, Cambridge Bioscience, UK). Recombinant human NT3 (R&D Systems, 267-N3) was used as a positive control for the experiment. Throughout the duration of the experiment, explants were supplemented with 10ng/ml of NGF (2.5S) in order to ensure their survival.

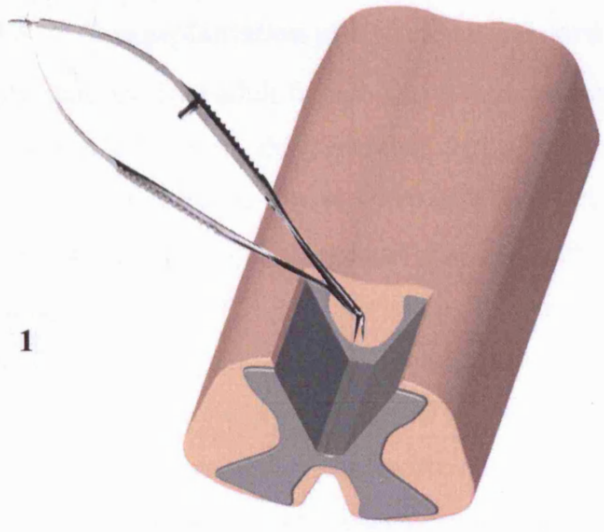
4.2.3 *In vivo* delivery of HSV1.pR19CMVrNT3

4.2.3.1 Corticospinal tract lesions

Lesions were performed on deeply anaesthetised adult female Lewis Rats, (≈200g). A longitudinal incision was made in the skin at the midline over the cervical spine. A second incision was made the superficial muscle layers and the underlying muscles were separated by blunt dissection. The C6 vertebra was exposed and a bilateral laminectomy performed using malleus scissors. The dura mater was carefully cut with micro-scissors to expose a small segment of the spinal cord. At this point the animals were injected with 2.2mg/Kg of Finadyne (Schering-Plough) for analgesia. With the aid of irridectomy scissors, an incision extending to the central canal was inflicted severing both the ascending dorsal columns and the dorsal corticospinal tracts (Figure 4.2.3-a). The dura and musculature was repaired using silk sutures and finally the skin was closed with Michel clips. Post-operatively, the animals were kept in a clean cage and monitored every hour for the first 6 hours and twice a day after the first day, until stable.

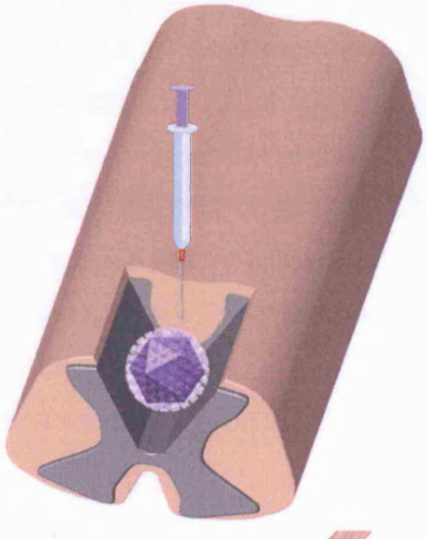
4.2.3.2 Spinal cord Microinjection of disabled HSV1 vectors

Spinal cords were exposed as described in the previous section. The pia matter was gently pierced with a fine needle in order to prevent crushing the tissue it protects. A 25 µl Hamilton syringe fitted with a fine needle (outer Ø 300 µm) was used to administer the vector. This was done by lowering the needle through the opening in the pia matter and 1.5 mm into the cord using the micromanipulator of a stereotaxic frame. After 5 min, the virus suspension was injected at a rate of 0.5 µl/min using a micropump. HSV1.pR19CMVrNT3 or HSV1.pR19CMVLacZ vector was delivered in two 2.5 µl aliquots, about 5 mm apart and either side of the lesion site, along the rostro-caudal axis of the C6-C7 segments. Following injection, the virus was allowed to diffuse for a further 10 minutes prior to removing the needle. Once the needle was removed, the dura, spine muscles and the overlaying skin was repaired with Michel clips.



1

CST Lesion



2

Virus Injection or Transplantation

3

BDA tracer

Figure 4.2.3-a: Schematic diagram of the CST lesion experiments.

The dorsal half of the spinal cord was transected bilaterally at the cervical level (**1**). In most experiments, any potentially therapeutic intervention such as vector administration or embryonic neural tissue transplantation (**2**) occurred at 1-month post lesion. BDA tracer was injected into the motor cortex (**3**) three days prior to the termination of the experiment. Spinal cords were sectioned at a thickness of 40μm and analysed via microscopy.

4.2.3.3 Transplantation of E14 rat spinal cord segments

Deeply anaesthetised adult female Lewis rats, received a CST lesion as described in section 4.2.3.1, with the exception that a 1.5-2 mm piece of gel foam was placed within the lesion site as soon as it was inflicted in order to prevent the creation of a large scar. Four weeks post injury the spinal cord was re-exposed and cleared of any scar tissue present at the lesion site by gently removing the piece of gel foam. This created a 2-3 mm gap within the lesion site. Whole E14 embryonic spinal cords were extracted as needed and dissected into 1 – 3 mm^3 segments which were then inserted within the newly created gap at the lesion site. Typically, one segment was necessary per animal. The dura was carefully sutured in order to keep the embryonic tissue in place. Muscle and skin were sutured in layers. Animals were kept in clean cages and 2 mg/Kg/day of Cyclosporin A (CsA) (Neoral®, Novartis, UK) was administered orally, one week prior to transplantation and throughout the four weeks following the transplant and vector administration when animals were terminated. CsA dosage was adjusted for the *Rattus Norvegicus* species and re-suspended in the animal's drinking water (Wolfensohn and Lloyd, 1998). The preparation was altered daily to ensure that no bacterial contamination occurred.

Note: Prof. P.N. Anderson performed the E14 spinal cord transplantations.

4.2.3.4 Tracing of CST fibres by biotin dextran processing

The cranium was exposed via a midline skin incision. Using a stereotaxic frame to hold a fine drill and to target the motor cortex, the brain was exposed by a hole through the cranium. The dura mater was delicately pricked to avoid crushing the tissue it protects. Using a 25 µl Hamilton syringe, 1 µl of the anterograde tracer 10% biotinylated dextran amine (BDA; 10,000MW, Molecular Probes) in PBS, was injected in the motor cortex for labelling the CST. The following co-ordinates were used for the CST (targeting the motor cortex at an angle of 10°): rostral-caudal 1-2 mm, medial-lateral 1-2 mm, dorsal-ventral 2.0 mm. Injections were made in 0.2 µl increments over two minutes. After a 10 min wait, to allow for the diffusion of the tracer, the needle was slowly withdrawn, muscles and the skin were sutured. Processing was carried out on free-floating sections for the

visualisation of BDA. Labelling was visualized by incubating the tissue for 1 hr with an avidin-peroxide complex (ABC kit; Vector Laboratories, Burlingame) diluted 1:500 in PBS containing 0.25% Triton X-100 (PBT). The tissue was rinsed three times for 10 min in PBS and then stained with 0.05% diaminobenzidine hydrochloride (DAB; Sigma, St. Louis, MO) and 0.01% H_2O_2 in PBS containing 0.01-0.02% nickel sulphate and 0.01-0.02% cobalt chloride. Sections were mounted on gelatinised slides and processed as already outlined in Chapter 2.

4.2.3.5 ELISA assay on vector secreted rNT3 *in vivo*

Deeply anaesthetised adult female Lewis rats received a unilateral CST lesion in the dorsal columns at the L5-L6 level. Three days later, animals were injected with $2.5x10^7$ pfu/ml of either HSV1.pR19CMVrNT3 (n=6) or HSV1.pR19CMVGFP (n=3) or 5 µl of DMEM (n=3) 3 days after lesioning. Three weeks later, animals were decapitated and a spinal cord segment 5 mm distal x 5 mm proximal to the injection site was quickly dissected and dipped in 4 volumes of ice cold DPBS to remove any excess blood. Tissue samples were immediately homogenised in lysis buffer (135 mM NaCl, 20 mM Tris/HCl pH 7.5, 1% Nonidet P40, 10% Glycerol, 1 mM PMSF, 1 µg/ml Leupeptin, 0.5 mM Sodium Orthovanadate) while kept at 4°C. Lysates were then centrifuged at 15,000g for 10 min at 4°C. The amount of total protein in each of the lysate supernatants was established using a commercially available BCA protein assay kit (BioRad) (Smith *et al.*, 1985). Free, total NT3 in the samples was detected using the NT3 E_{max}® Immunoassay system (Promega) according to manufacturer's instructions and expressed in pg/mg of total protein.

4.3 Results

4.3.1 Functional characterisation of vector HSV1.pR19CMVrNT3

Cloning of the *Ntf3* gene into the pGEM5.pR19CMV plasmid in place of GFP was confirmed by sequencing the region following the CMV promoter. The ability of the HSV1.pR19CMVrNT3 vector to express the transgene was assessed both *in vivo* and *in vitro*. The correct molecular characteristics of the vector were assessed by ensuring that the white recombinant phenotype observed in infected cells was due to the correct insertion of the pR19CMVrNT3 expression cassette into each of the two endogenous LAT regions (Figure 4.3.1-a). This was confirmed by Southern Blot analysis on three recombinant plaques. As shown in Figure 4.3.1-b (panel **A**), all three plaques appear positive for the presence of the transgene, with an ≈800bp band (Maisonpierre *et al.*, 1991) being present following *XhoI* digestion of the plaque viral DNA. This band corresponds to the one obtained via the same restriction on pBKS-rNT3 and pGEM5.pR19CMVrNT3 plasmid DNA which were used as positive controls.

To assess the capability of the HSV1.pR19CMVrNT3 vector to transduce cells, $1x10^6$ BHK cells were infected at an MOI of 1 with either HSV1.pR19CMVGFP (n=2) or HSV1.pR19CMVrNT3 (n=2 per time point). Supernatants were collected at 8 hours in the case of HSV1.pR19CMVGFP (control) and at 2, 4 and 8 hour time points in the case of HSV1.pR19CMVrNT3 infected cells (n=2 per time point). Conditioned media was analysed for the presence of secreted rNT3 using standard ELISA or Western blotting techniques. ELISA analysis of the 8 hour time point conditioned media confirmed that HSV1.pR19CMVrNT3 is capable of transducing non complementing cells to express the transgene (Figure 4.3.1-d, panel **A**). Expression is efficient with 13.5 ng ±1.3 (Mean ± SEM) of rNT3 produced per $1x10^6$ infected cells over an 8 hour period (P=0.00004). Even though this assay confirms the presence of rNT3, it does not provide any information about whether the protein detected corresponds to the mature form of the active protein or not. It was therefore necessary to assess the molecular weight migration profile of the bands recognised by an NT3 specific antibody.

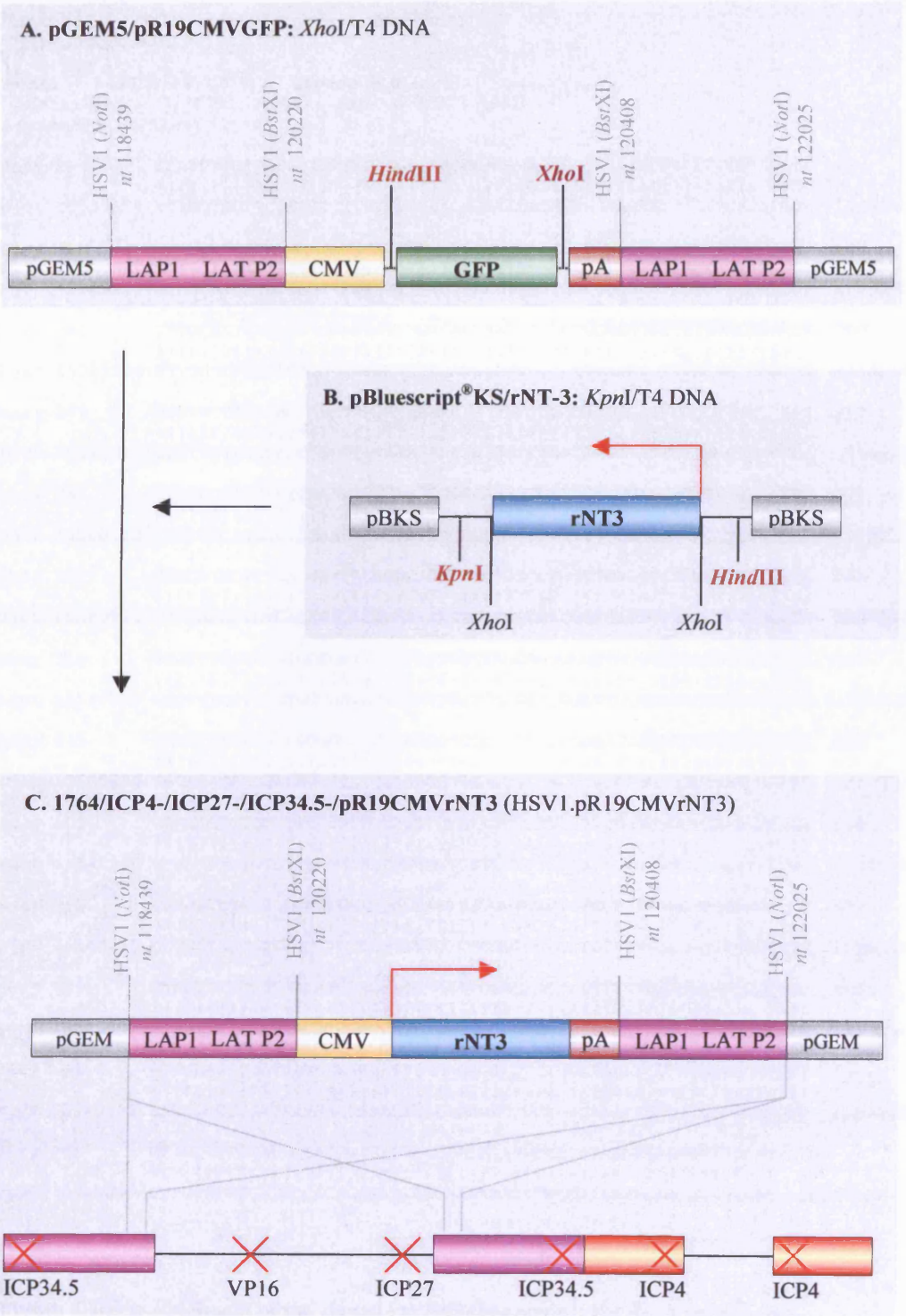


Figure 4.3.1-a: Construction of vector HSV1.pR19CMVrNT3.

```
>ref|NW_047696.2|Rn4_WGA2219_3 Rattus norvegicus chromosome 4 genomic contig
          Length=39585501

Features in this part of subject sequence:
   neurotrophin 3

Score = 1494 bits (777),  Expect = 0.0
 Identities = 777/777 (100%), Gaps = 0/777 (0%)
 Strand=Plus/Plus

Query 21       TCATGTTCTTCCGATTTTTCTTGACAAGGCACACACACAGGAAGTGTCTATTCGTATCCA  80
               ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct 15539870 TCATGTTCTTCCGATTTTTCTTGACAAGGCACACACACAGGAAGTGTCTATTCGTATCCA  15539929

Query 81       GCGCCAGCCTACGAGTTTGTTGTTTTCTGAAGTCAGTGCTCGGACGTAGGTTTGCGACGT  140
               ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct 15539930 GCGCCAGCCTACGAGTTTGTTGTTTTCTGAAGTCAGTGCTCGGACGTAGGTTTGCGACGT  15539989

Query 141      TTTGCACTGAGAGTTCCAGTGTTTGTCATCAATCCCCCTGCAACCGTTTTTGACTGGCCT  200
               ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct 15539990 TTTGCACTGAGAGTTCCAGTGTTTGTCATCAATCCCCCTGCAACCGTTTTTGACTGGCCT  15540049

Query 201      GGCTTCTTTACACCTCGTTTCATAAAAATATTGTTTCACAGGAGAGTTGCCGGTTTTGAT  260
               ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct 15540050 GGCTTCTTTACACCTCGTTTCATAAAAATATTGTTTCACAGGAGAGTTGCCGGTTTTGAT  15540109

Query 261      CTCTCCCAACACTGTAACCTGGTGTCCCCGAATGTCAATGGCTGAGGACTTGTCGGTCAC  320
               ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct 15540110 CTCTCCCAACACTGTAACCTGGTGTCCCCGAATGTCAATGGCTGAGGACTTGTCGGTCAC  15540169

Query 321      CCACAGGCTCTCACTGTCACACACTGAGTACTCTCCTCGGTGACTCTTATGCTCTGCATA  380
               ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct 15540170 CCACAGGCTCTCACTGTCACACACTGAGTACTCTCCTCGGTGACTCTTATGCTCTGCATA  15540229

Query 381      GCGTTTCCTCCGTGGTGATGTTCTATTGGTTACCACCGGGTTGCCCACATAATCTTCCAT  440
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Sbjct 15540230 GCGTTTCCTCCGTGGTGATGTTCTATTGGTTACCACCGGGTTGCCCACATAATCTTCCAT  15540289

Query 441      TAGATATAAGGGAGGGGGCTCCAAAGGGGTGCTGTCACTCAGCAGGACCCGGGGTGAATT  500
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Sbjct 15540290 TAGATATAAGGGAGGGGGCTCCAAAGGGGTGCTGTCACTCAGCAGGACCCGGGGTGAATT  15540349

Query 501      GTAGCGTCTCTGTTGCCGTAGTAGTTCTGTGTCTGTTGCAATCATCGGCTGGAATTCTGA  560
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Query 561      CCTGGTGGCCTCTCCCTGCTCTGGTTCTCTGGGTGCCTCTGCTTTGGGCAGGGTGCTCTG  620
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Sbjct 15540410 CCTGGTGGCCTCTCCCTGCTCTGGTTCTCTGGGTGCCTCTGCTTTGGGCAGGGTGCTCTG  15540469

Query 621      GTAATTTTCCTTAACATCTACCATCTGCTTGGAGAGCTTGTTTTTCAAGATATCCGCCTG  680
               ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
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Query 681      GATCAACTTGATAATGAGGGAATTGAGAGAGTCTTCTGGCAAACTCCTTTGATCCATGTT  740
               ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct 15540530 GATCAACTTGATAATGAGGGAATTGAGAGAGTCTTCTGGCAAACTCCTTTGATCCATGTT  15540589

Query 741      GTTGCCTTGGATGCCACGGAGATAAGCAAGAAATATCACATAAAACAAGATGGACAT  797
               |||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct 15540590 GTTGCCTTGGATGCCACGGAGATAAGCAAGAAATATCACATAAAACAAGATGGACAT  15540646
```

Figure 4.3.1-b: Alignment of the cloned rat *Ntf3*gene against the draft rat genome.

The cloned rat *Ntf3* sequence shown above is identical to that of contig NW_047696.2 from the *Rattus Norvegicus* genome.

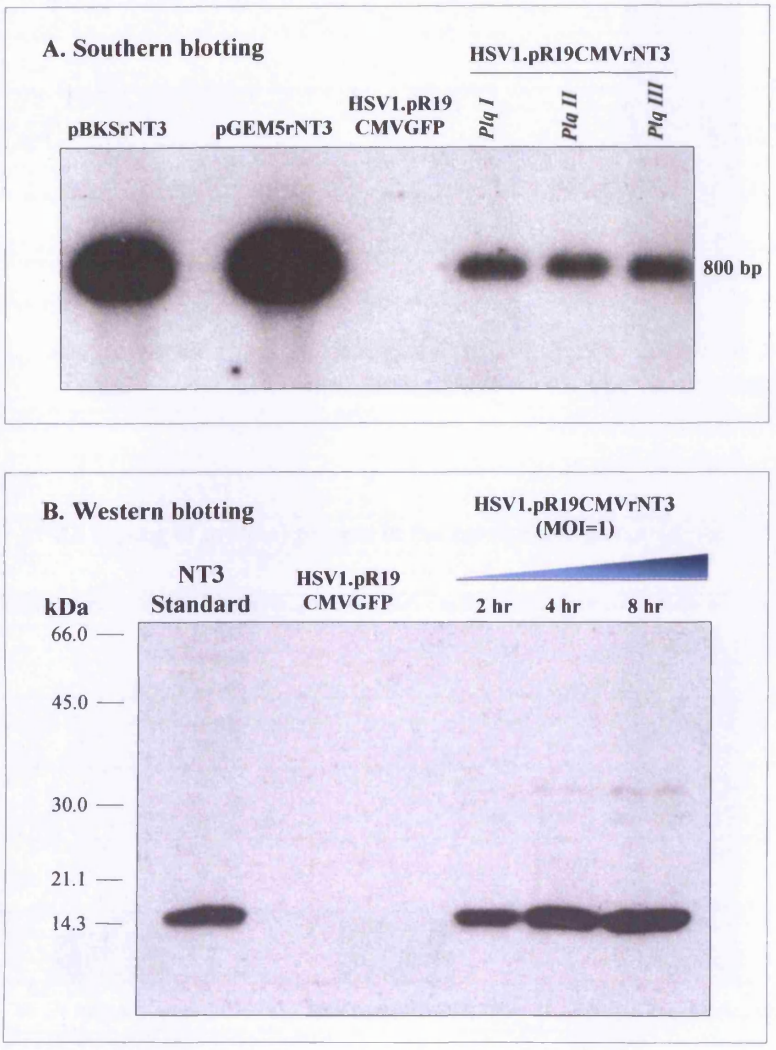


Figure 4.3.1-c: Functional characterisation of HSV1.pR19CMVrNT3.

A. Southern blotting on three purified recombinant plaques of HSV1.pR19CMVrNT3 vector. pBKSrNT3 and pGEM5pR19CMVrNT3 were used as positive controls while HSV1.pR19CMVGFP was used as a negative control. The only band recognised from the rNT3 probe (pKS-NT3, *XhoI* digest) corresponds to the rNT3 gene (≈800bp).

B. Western blot analysis of conditioned media from HSV1.pR19CMVGFP (n=2) or HSV1.pR19CMVrNT3 (n=2 per time point) infected BHK cells, probed with Rabbit polyclonal anti-rat NT3. Recombinant rNT3 was loaded at 10 µg/ml to act as a positive control. Conditioned media obtained from $1x10^6$ BHK cells, infected with HSV1.pR19CMVGFP at the same MOI did not produce any bands that cross-reacted with the primary antibody used, endorsing its specificity to rNT3.

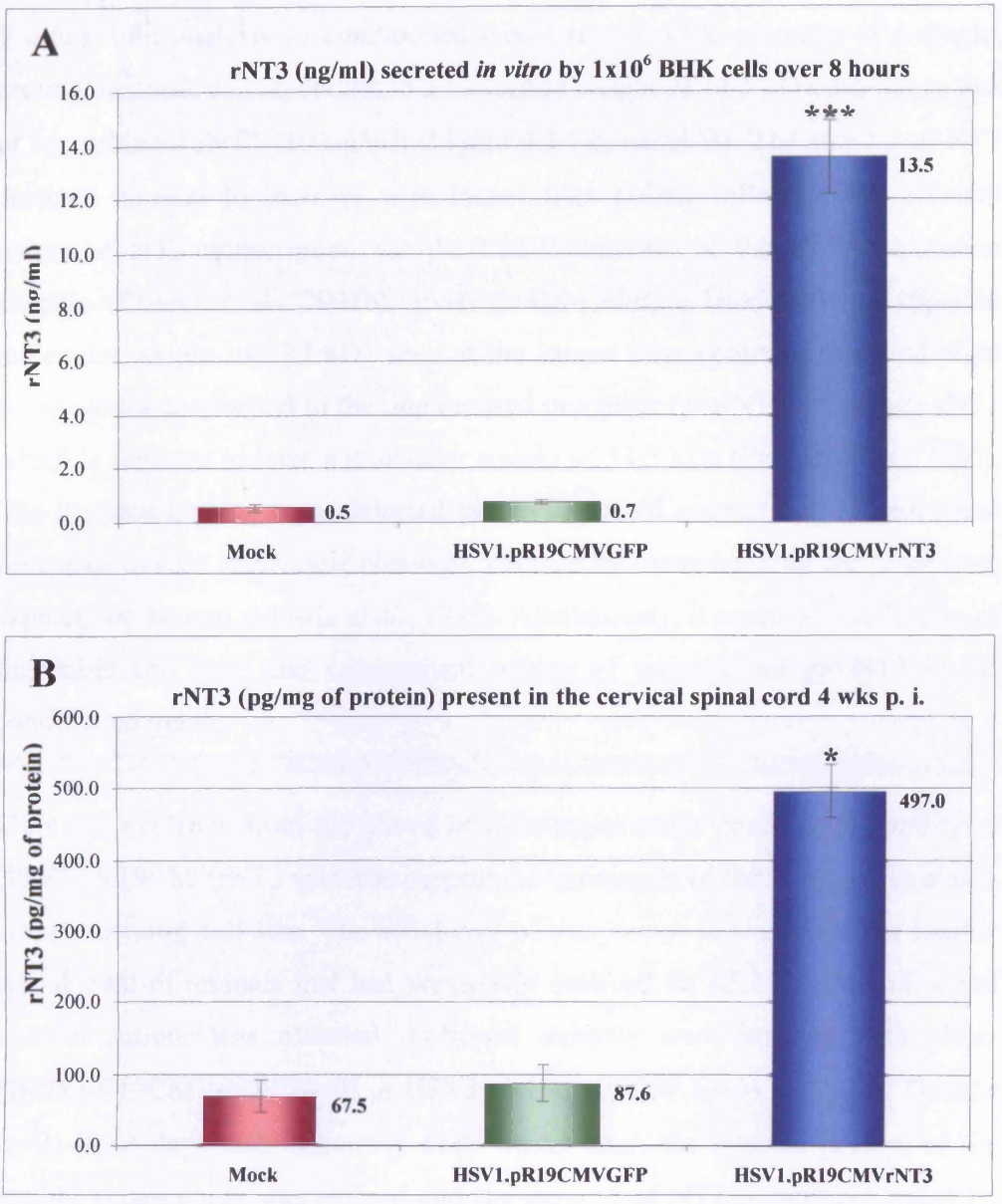


Figure 4.3.1-d: *In vitro* & *in vivo* expression of rNT3 via HSV1.pR19CMVrNT3.

In vitro and *in vivo* ELISA assay using the Promega NT3 E_{max}® Immunoassay system.

A. Supernatants from $1x10^6$ BHK cells either uninfected (Mock, n=3) or previously infected with HSV1.pR19CMVrNT3 (n=6) or HSV1.pR19CMVGFP (n=2) vectors at an MOI of 1, were analysed in triplicate. HSV1.pR19CMVrNT3 infected cells appear to efficiently express and secrete the transgene with $1x10^6$ BHK cells producing 13.5ng/ml ± 1.3 (Mean ± SEM O.D.) of vector encoded rNT3, over 8 hours. Corresponding supernatants from cells infected with HSV1.pR19CMVGFP vector demonstrate no interference with the detection of HSV1.pR19CMVrNT3 vector delivered rNT3 (Student's T-test: ***P<0.001, compared to HSV1.pR19CMVGFP infected cells).

B. L5-L6 spinal cord segment was inoculated with either $2.5x10^6$ pfu of HSV1.pR19CMVrNT3 (n=3) or HSV1.pR19CMVGFP (n=3) vector or with 5 µl of DMEM (Mock, n=3). Three weeks after inoculation, spinal cords were assayed in triplicate. The amount of rNT3 produced in transduced spinal cords was found to be 497 pg/mg of protein ± 19.4 (Mean ± SEM O.D.) (*P<0.05 compared to HSV1.pR19CMVGFP treated animals), which is significantly higher than that found in either control or Mock-injected spinal cords.

Western blot analysis of conditioned media revealed the presence of a single, prominent band, corresponding to a molecular weight of 14.5 kDa, similar to that of recombinant rNT3 (10 μg/ml) (Figure 4.3.1-c, panel **B**). The amount of NT3 detected appears to increase with longer time points, reflecting the efficient nature of NT3 transcription via the CMV promoter of the pR19 expression cassette (Lilley *et al.*, 2001b). Even though faint, a band with an apparent molecular weight of ≈35 kDa seen at the longer time points of four and eight hours, could correspond to the unprocessed precursor (pro-NT3) of mature rNT3 which is reported to have a molecular weight of 33.5 kDa (Farhadi *et al.*, 2000). The increasing amounts of detected pro-rNT3, could suggest that at least some precursor evades proteolytic cleavage, perhaps by overwhelming the processing capacity of the cell (Mowla *et al.*, 1999). Alternatively, it could be a reflection of inevitable cell lysis and concomitant release of unprocessed pro-NT3 in the conditioned media.

Thus far, evidence from the above *in vitro* experiments confirms the ability of HSV1.pR19CMVrNT3 vector to support the expression of the transgene in a non-complementing cell line. The efficiency of this vector to transduce the lumbar spinal cord of animals that had previously received an L5-L6 unilateral dorsal column lesion, was assessed. Lesioned animals were injected with either HSV1.pR19CMVrNT3 (n=3) or HSV1.pR19CMVGFP (n=3) or 5 μl of DMEM (n=3) three days after lesioning. Four weeks later, the injected portion of the lumbar spinal cords was excised and the amount of NT3 present estimated by ELISA assay. As shown in Figure 4.3.1-d (panel **B**), the amount of NT3 was found to be 497pg/total mg of protein ± 19.4 (Mean ± SEM) in HSV1.pR19CMVrNT3 treated animals, which is significantly different (P=0.012) to that measured in control or mock treated animals. The 4-week time point, selected for this analysis served the purpose of demonstrating that this was a viable point at which to assess any potential regeneration in experiments involving HSV1.pR19CMVrNT3. The amount of rNT3 detected in injected spinal cords may at first appear rather low when considering the strong expression levels obtained when delivering the marker gene LacZ via the same backbone. However, LacZ is an intracellular enzyme that, lacking any suitable

signals, is not secreted, as is the case for NT3. Instead, it accumulates within the cell for the duration of the experiment and can distort the quantity of transgene converted to actual protein. In addition, since it is an enzyme, the depth of blue colour also depends on the amount of substrate available rather than just the levels of transgene expression. It is therefore an unreliable marker in terms of actual gene expression levels and it would be misleading to assume that the amount of NT3 protein produced by infected cells is comparable to that obtained for LacZ at similar time points.

Noticeably, the amount of NT3 present is approximately half of that found when adenoviral (Blits *et al.*, 2000) or adeno-associated (Blits *et al.*, 2003) vectors encoding NT3 under the control of a CMV promoter, are used either to transduce 293 cells in culture (Blits *et al.*, 2000; Blits *et al.*, 2003) or injected into adult rat spinal cords (Dijkhuizen, 1999). Compared to a differently disabled but HSV1 based vector however the amount of NT3 produced *in vivo* and *in vitro*, reported at 100 pg/mg of protein and 15 $\mu g/10^3$ cells respectively, is comparable in scale to the one achieved with vector HSV1.pR19CMVrNT3 (Chattopadhyay *et al.*, 2002). The disparity noted between adenoviral and HSV1 based vectors could be due to the ability of HSV1.pR19CMVrNT3 to specifically transduce neurons compared to an adenoviral vector that can infect non-neuronal populations as well (Dijkhuizen, 1999; Blits *et al.*, 2000). NT3 secreted from non-neuronal cells could contribute to the total amount detected by the ELISA. To complicate matters even more, vectors are often utilised at different pfu, which is in turn determined by their overall toxicity *in vivo*. Differences in pfu would directly impact on the overall transgene expression levels thus complicating any direct comparison. Furthermore, the procedure used to measure the total amount of NT3 present in spinal cord samples is prone to variations as it depends on the thoroughness of the operator and speed of tissue extraction. It is therefore very difficult to safely draw any solid conclusions regarding the relative ability of the HSV1.pR19CMVrNT3 vector to induce a biological effect based on its ability to transduce a target population. It is also unsafe to draw any definitive conclusions regarding this vector's efficiency compared to that achieved with other vector systems used to deliver transgenes in the CST.

The ability of HSV1.pR19CMVrNT3 vector to deliver biologically active NT3 was evaluated on E14 lumbar DRG explants. At E14, DRGs have been shown to contain a subpopulation of NT3 responsive neurons (Maisonpierre *et al.*, 1990b; Hory-Lee *et al.*, 1993) which could be stimulated to extend neurites in the presence of biologically active NT3 (Crowley *et al.*, 1994; Smeyne *et al.*, 1994). The ability of vector-encoded rNT3 to induce such a biological response was assessed by means of treating the explants with conditioned media obtained from transduced, non-complementing BHK cells. Collagen embedded E14 explants were supplemented daily either with recombinant human NT3 (n=2 DRGs per well) or with media collected from $1x10^6$ BHK cells, previously infected with either HSV1.pR19CMVGFP (n=3) or HSV1.pR19CMVrNT3 (n=3) at an MOI of 1. Conditioned media was obtained fresh each day and it was briefly clarified in order to remove cellular debris. Following three days of treatment, explants were fixed and immunostained with TUJ1 neurofilament antibody.

Figure 4.3.1-e (panel **C**) clearly demonstrates that vector-encoded rNT3 is capable of inducing the extension of a dense network of neurites radiating from E14 DRG explants treated with HSV1.pR19CMVrNT3 conditioned media. Moreover, vector encoded rNT3 appears to induce the outgrowth of thick neurite bundles which correspond to an NT3-specific biological response (Davies, 2000). It has previously been reported that mice lacking NT3 during development develop severe sensory neuron deficits in proprioceptive DRG neurons and demonstrate an inability to perform coordinated motor tasks (Farinas *et al.*, 1994; Klein *et al.*, 1994; Tuttle *et al.*, 1995). Neurite elongation appears to be more robust than the growth promoted when using 50 ng/ml of recombinant NT3 (panel **B**). This is not surprising as recombinant NT3 was not replenished for the duration of the experiment, unlike the HSV1.pR19CMVrNT3 conditioned media, which was replenished on a daily basis. Contrary to the dense growth observed in HSV1.pR19CMVrNT3 treated explants, bathing them in HSV1.pR19CMVGFP conditioned media mediates minimal neurite outgrowth. This experiment confirms that vector-encoded rNT3 is biologically active and capable of inducing a growth promoting effect, at least *in vitro*, and provides the basis for further in vivo regenerative attempts targeting the chronically injured corticospinal tract.

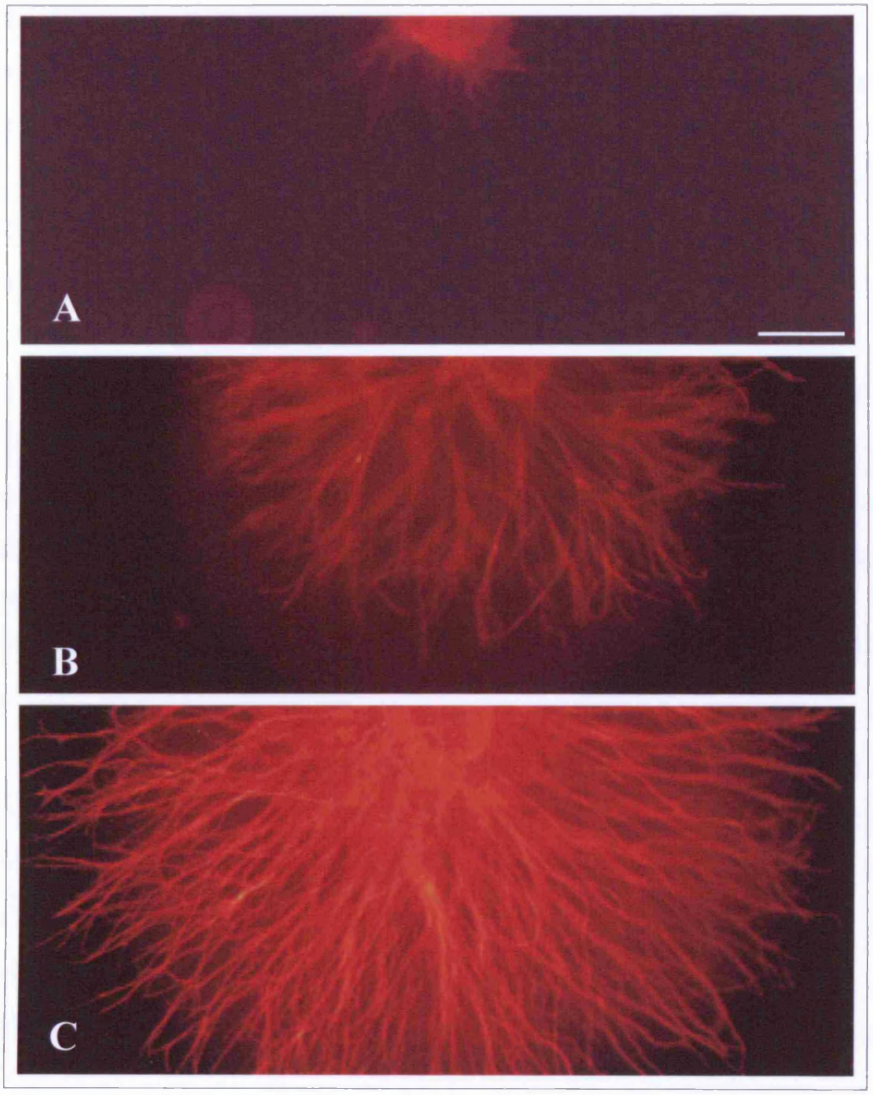


Figure 4.3.1-e: Biological assay for vector encoded rNT3 on E14 rat DRGs.

All E14 rat DRG explants (section 4.2.2.7) were supplemented with 10 ng/ml of NGF (2.5S) on which they depend on for survival at that age (Crowley *et al.*, 1994; Smeyne *et al.*, 1994). TUJ1 immuno-staining on collagen embedded E14 DRGs treated with either **A**, **B** or **C**.

A. Negative control (not infected) E14 explants supplemented with conditioned media from HSV1.pR19CMVGFP $1x10^6$ infected BHK cells, display minimal neurite outgrowth.

B. Positive control explants supplemented with 50 ng/ml of recombinant human NT3. (Maximal activity is reported at 100ng/ml, R&D systems protocol).

C. Explants supplemented with media from HSV1.pR19CMVrNT3 $1x10^6$ infected BHK cells at an MOI of 1 display a dense halo of thick neurite bundles.

Scale Bar: 500μm, applies to all figures.

4.3.2 Effect of vector encoded rNT3 on CST regeneration

This study aimed to address whether HSV1.pR19CMVrNT3 is capable of promoting regeneration in the chronically injured spinal cord model. For our purposes, a chronic injury refers to an injury inflicted at least one month prior to any intervention and it is under these circumstances that the HSV1.pR19CMV based vectors have been evaluated in transducing the CST tract. In Chapter 3, it was demonstrated that simultaneous proximal and distal injection of the vector allowed the best transduction to occur in the chronic CST lesion model. Furthermore, it was shown that transgene expression persists for at least one month following inoculation (Lilley *et al.*, 2001b). However, it also became evident that this generation of vectors did not have the ability to sustain transgene expression in the injured or uninjured CST neurons. Whether this method of administration would enable vector HSV1.pR19CMVrNT3 to mediate regeneration in the chronic CST model was explored in this study.

$2.5x10^6$ pfu of HSV1.pR19CMVrNT3 (n=8) or HSV1.pR19CMVLacZ (n=3) vector were injected both proximal and distal to the lesion site at the C6 level, in pre-lesioned animals (section 4.2.3.1). Four weeks following vector inoculation, very few axons can be found within the lesion site of HSV1.pR19CMVLacZ treated animals, most of which appear to have retraction bulbs, indicative of abortive growth (Figure 4.3.2-a, panel **A**). In three of the eight HSV1.pR19CMVrNT3 vector treated animals some evidence of proximal sprouting can be seen with only a few axons extending into the lesion site (Figure 4.3.2-a, panel **B**). However, proximally sprouting axons appear to follow an irregular route, seemingly turning away from the edge of the lesion site. The number of axons found within the lesion site is marginally higher than that observed in animals treated with HSV1.pR19CMVLacZ vector only (control). Also, abortive regeneration appears less prominent than that observed in control animals. Thus, it would be reasonable to conclude that HSV1.pR19CMVrNT3 treatment appears to minimally enhance the number of regenerating, BDA positive axons and reduce the number of retraction bulbs found within the lesion site, compared to HSV1.pR19CMVLacZ treated animals.

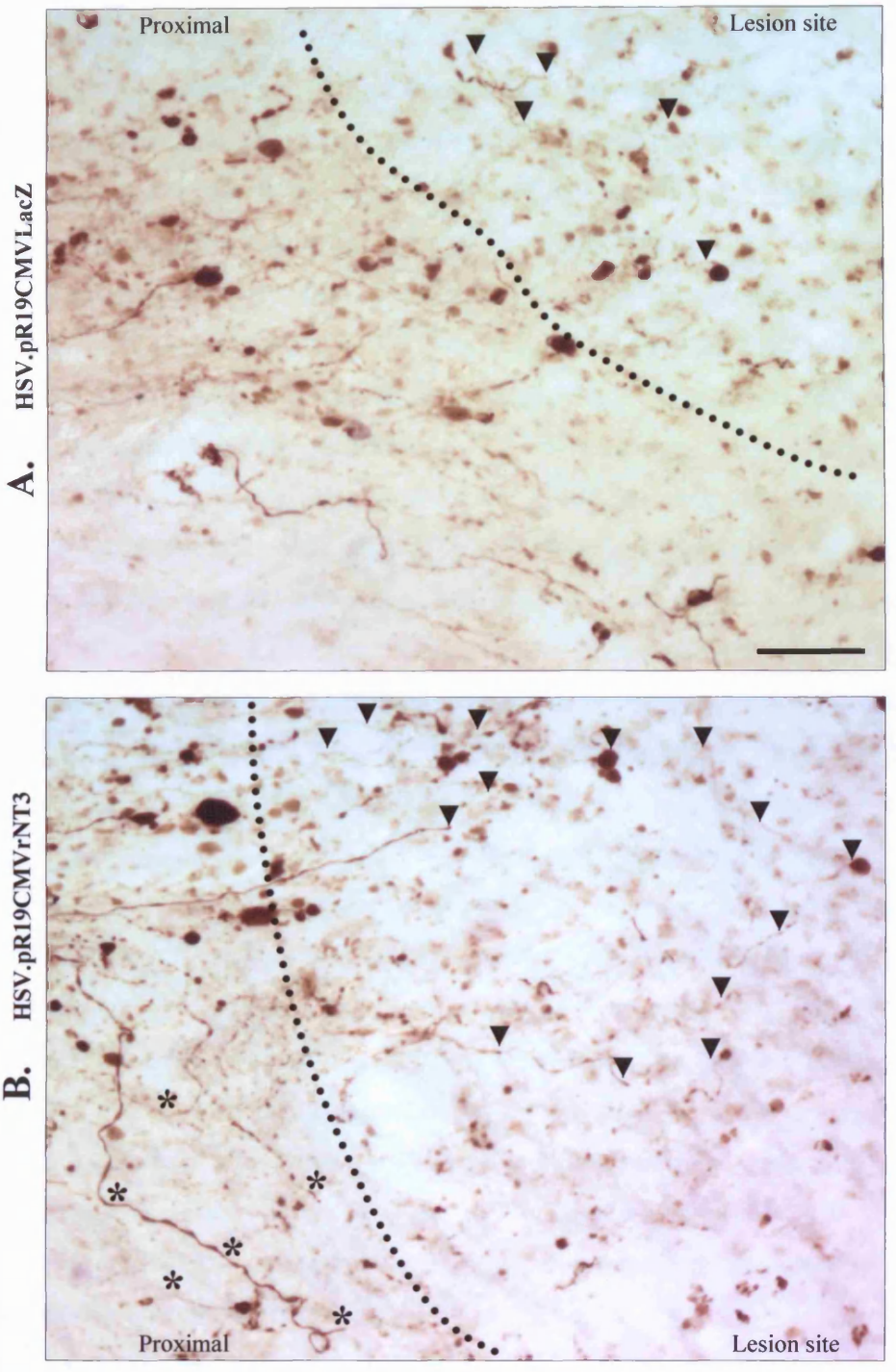


Figure 4.3.2-a: HSV1.pR19CMVrNT3 administration minimally induces CST regeneration.

Longitudinal sections of spinal cord showing BDA labelled CST axons at the site of a CST lesion at the C6 level, made one-month previously. HSV1.pR19CMVLacZ vector (**A**) or HSV1.pR19CMVrNT3 vector (**B**) were injected both proximal and distal to the lesion site. **A.** Very few BDA labelled CST axons are present within the lesion site, most with retraction bulbs (▼). Low level, abortive sprouting can be seen proximally to the lesion. **B.** Three CST axons show low level sprouting proximally as well as into the lesion site. Proximal sprouting axons appear to follow an irregular route (*). The dotted line demarcates the proximal boundaries of the lesion site. Scale bars: 50μm, applies to **A** and **B**.

The results from the three animals described here represent the best result obtained from a series of similar experiments that demonstrated no apparent anatomical difference between control and rNT3 treated animals. This distinct inability of vector delivered rNT3 to mediate any significant regeneration was noticed early in this study and prompted us to repeat this attempt in order to rule our any potential operator errors. The main error considered was the possibility of not delivering the correct amount of viral suspension, thus altering the total pfu. Over the course of these studies it became clear that the injection method and animal survival were the Achilles heel of these experiments. After having repeated this experiment numerous times (Total number of animals used HSV1.pR19CMVLacZ: n=6 and HSV1.pR19CMVrNT3: n=18) it became apparent that the lack of regeneration was unlikely to be due to an experimental discrepancy but rather reflects an underlying inability of vector-encoded rNT3 to promote any meaningful regeneration, anatomically at least, in the chronically injured CST. In addition to the simultaneous inoculation of the vector both proximal and distal to the lesion site described here, the effect of a single injection of HSV1.pR19CMVrNT3 vector either proximally or distally had previously been addressed (results not shown). This was done in order to explore the possibility that vector administration had to reflect the role NT3 will have to fulfil as a regeneration-promoting factor. Firstly, we examined the possibility that rNT3 had to be delivered distally in order for it to act as a chemo-attractant for the injured CST axons. Several reports suggest that for CST axons to regenerate they must have a guided growth towards an increasing concentration gradient of the neurotrophic factor (Bradbury *et al.*, 1999). Therefore NT3 should be placed distally to the lesion so that axons are encouraged to transverse the lesion site and exit caudally towards the source of the neurotrophin. For sole delivery distally to the lesion site to be successful, the vector must be administered distally enough so that uninjured neurons in the spinal cord become transduced and secrete NT3, while at the same time close enough to the injured axons so that local virally-induced rNT3 over-expression can positively impact the axotomised CST fibres. It has proved technically very difficult to correctly deliver such an injection in the chronically injured CST model. Even though a stereotactic frame was used, demarcation of the lesion site in the living animal is often less that

straightforward. Secondly, we examined the possibility that vector encoded rNT3 had to be delivered in close proximity to the injured CST axons in order for them to have maximum stimulation towards regeneration. Proximal administration needs to target local neuronal somata that would produce an *in situ* pool of rNT3 that would attract axotomised CST axons. Since HSV1.pR19CMVrNT3 vector preferentially infects neurons and NT3 can be transported bi-directionally (DiStefano *et al.*, 1992; Altar and DiStefano, 1998), it was expected that either method would at least demonstrate some effect, if not regenerative then at least have an impact on the number of retracting bulbs present following axotomy. Disappointingly, none of these treatments produced any effect.

Even though frustrating, the inability of sole vector mediated NT3 administration to promote any meaningful CST regeneration is surprising. Perhaps the total lack of an effect suggests that delivering the vector in a single location might be an inefficient and irreproducible means of administration. It is logical to suggest that a single administration, compliant with such stringent criteria is just too difficult to achieve in a reproducible and reliable manner. For example, wrongful administration of the vector too close to the lesion site would result in minimal expression of rNT3. This is because HSV1.pR19CMV based vectors do not transduce glia or other non-neuronal cells, potentially predominating near the lesion. This negative result prompted us to carry out the double delivery experiments shown here where the vector was delivered both proximally and distally to the lesion site. As it was demonstrated in Chapter 3, vectors based on the HSV1.pR19CMV backbone were unable to directly transduce CST neurons after inoculation, either into upper motor neurons in layer V of the adult rat motor cortex or after delivery into the spinal cord. This appeared to be specific to the CST since targeting the rubrospinal tract produces significant retrograde labelling of red nucleus neurons (Chapter 5). The major advantage of HSV1 based vectors of being able to be retrogradely transported from the site of inoculation to neuronal somata could not be exploited. At the time these experiments were conducted however, vector HSV1.pR19CMV was the optimum vector for regenerative studies in terms of achieving highly efficient delivery while being minimally cytotoxic.

4.3.3 Combining rNT3 delivery with E14 spinal cord transplants

In this set of experiments we set out to explore whether HSV1pR19CMV - mediated delivery of rNT3 is able to induce regeneration in the presence of an E14 spinal cord segment, transplanted within the lesioned spinal cord of adult rats. This experimental approach was based on the Bregman *et al.* (1998) paradigm in which transplanted E14 spinal cord segments within the lesion site appeared to work synergistically with co-administered NT3 to induce CST, raphaespinal and coeruleospinal axonal regeneration through the transplant (Bregman *et al.*, 1998). Without acute neurotrophic administration (1 mg/ml), regenerating CST axons are confined to the host/transplant border (Bregman *et al.*, 1989). In a later study Bregman and colleagues (2002) went on to show that delaying transplantation of embryonic tissue and neurotrophin administration for 2 weeks following a transecting lesion, leads to an increase in the number of supraspinal and propriospinal regenerating axons as well as some recovery of locomotor function (Bregman *et al.*, 2002). Based on the above series of experiments, this study employed HSV1.pR19CMVrNT3 vector as a means of delivering biologically active NT3 in conjunction with E14 spinal cord segment transplants. Firstly, adult rats received a CST lesion with bi-lateral sectioning of the dorsal columns. Two weeks later, the lesion site was cleaned of scar tissue and all animals received one or two segments of E14 spinal cord. Prof. Patrick Anderson (Dept. of Anatomy & Developmental Biology, UCL) performed the CST lesions and subsequent grafting of embryonic spinal cords.

Transplanted animals received $2.5x10^6$ pfu of either control HSV1.pR19CMVLacZ vector (n=4) or HSV1.pR19CMVrNT3 vector (n=8). Four weeks later, animals were terminated and examined for any signs of regeneration. The boundaries of the transplant can be identified by the large number of dark oblong nuclei found around the perimeter of the lesion site. These could possibly correspond to fibroblasts and infiltrating macrophages (Figure 4.3.3-a, panel **C**). However, the boundaries of the lesion and injection sites were harder to define. For the purposes of this study the lesion site was identified by microscopy as lacking any tissue coherence with loose, often acellular patches where axons were absent or degenerating.

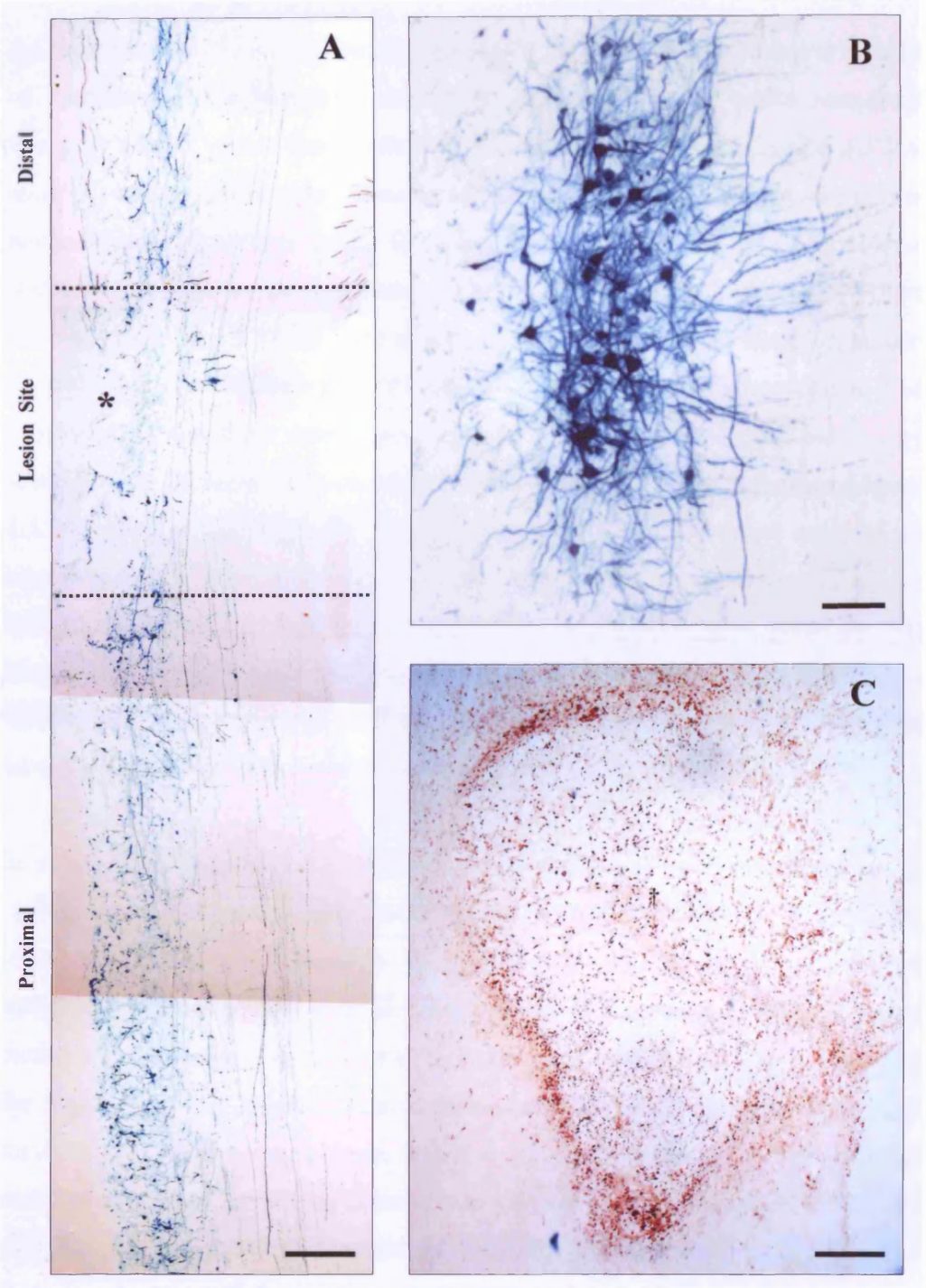


Figure 4.3.3-a: Combined HSV1.pR19CMVLacZ & E14 spinal cord treatment.

Efficient LacZ transduction in spinal cord, neurons following simultaneous vector injection and transplantation of E16 spinal cord segments into a C6, dorsal column lesion site made 4 weeks previously (*). Vector ($2.5x10^6$ pfu) was injected within and proximally to the lesion site. Large numbers of LacZ transduced neurons are shown in a low magnification micrograph of a longitudinal section through the lesion site as well as proximally and distally to it (**A**). Ventral motor neurons near the injection site appear healthy (**B**). The transplant (†) in the lesion site can be distinguished from the rest of the tissue (**C**).

Scale bars: **A**=500μm; **B**=200μm; **C**=500μm.

As demonstrated in control animals, the HSV1.pR19CMVLacZ vector is capable of maintaining the long-term expression of the transgene while retrograde transport of the vector has resulted in widespread expression (Figure 4.3.3-a, panel **A**) of the marker gene. Transduced neurons within the injection site appear normal (panel **B**) at four weeks following inoculation. Apart from two control animals examined for the expression of LacZ (one of which is shown in Figure 4.3.3-a, panel **A**), all others were processed for the detection of the BDA tracer. Animals that received vector HSV1.pR19CMVLacZ in conjunction with an E14 transplant, demonstrate some sprouting proximal to the transplant and a very small number of axons, all with retraction bulbs, just within the transplant (Figure 4.3.3-b, panel **A** and Figure 4.3.4-c, panel **A**). In animals that had received an inoculum of HSV1.pR19CMVrNT3 vector, more regenerating CST axons appear to enter the transplant (Figure 4.3.3-b, panel **B** and Figure 4.3.3-c, panel **B**). The longest regenerating axon was placed at 600 μm within the lesion site (Figure 4.3.3-c, panel **C**). Moreover, CST axons found proximally as well as within the transplant appear to have fewer retraction bulbs than in control animals.

In panel **C** of Figure 4.3.3-b, a more ventral section of the transplanted spinal cord is shown. In this section no fibres can be seen entering the transplanted tissue but rather they bypass it by growing around the boundaries of the transplant. The possibility of these being spared CST axons that were displaced during transplantation (Steward *et al.*, 2003) was discounted due to the fact that the lesion site was cleared prior to transplantation. Even if CST axons had survived the initial dorsal column lesion it is highly unlikely they would have survived the procedure of scar clearing and subsequent transplantation. It is more plausible that axons in this case did not cross the lesion site due to the fact that there is a larger acellular cavity present perhaps because the transplant did not extend so ventrally and the lesion site has refilled with a loose network of scar tissue. Therefore, the lack of substrates within the lesion site means that it cannot act as a support medium for CST axons that have been induced to regenerate.

Figure 4.3.3-b: HSV1.pR19CMVrNT3 & E14 spinal cord transplantation treatment.

BDA labelled (light brown) CST axons present in longitudinal spinal cord sections of rats that had received a simultaneous vector injection ($2.5x10^6$ pfu of HSV1.pR19CMVrNT3) and E14 segment the transplantation within the site of a dorsal hemisection performed one month previously. Control animals (**A**) that were injected with vector HSV1.pR19CMVLacZ ($2.5x10^6$ pfu) demonstrate a minimal regenerative response with very few axons entering the lesion site and none transversing the transplant (shown in Figure 4.3.3-c, panel **A**). In HSV1.pR19CMVrNT3 treated animals (**B** & **C**), many regenerating axons (▼) can be seen within the lesion site as well as transversing the transplant (Section B, shown Figure 4.3.3-c, panels **Bi** & **Bii**). This effect was observed in four of the six animals receiving this treatment. Panels **B** & **C** show sections from the same animal with section **C** being more ventral to section **B.** Axons appear to pass through the lesion site in the more dorsal layers (**B**). The dotted lines demarcate the proximal (left) and distal (right) boundaries of the lesion site.

Scale Bars: **A**=200μm; **B**=500μm, applies to **C**.

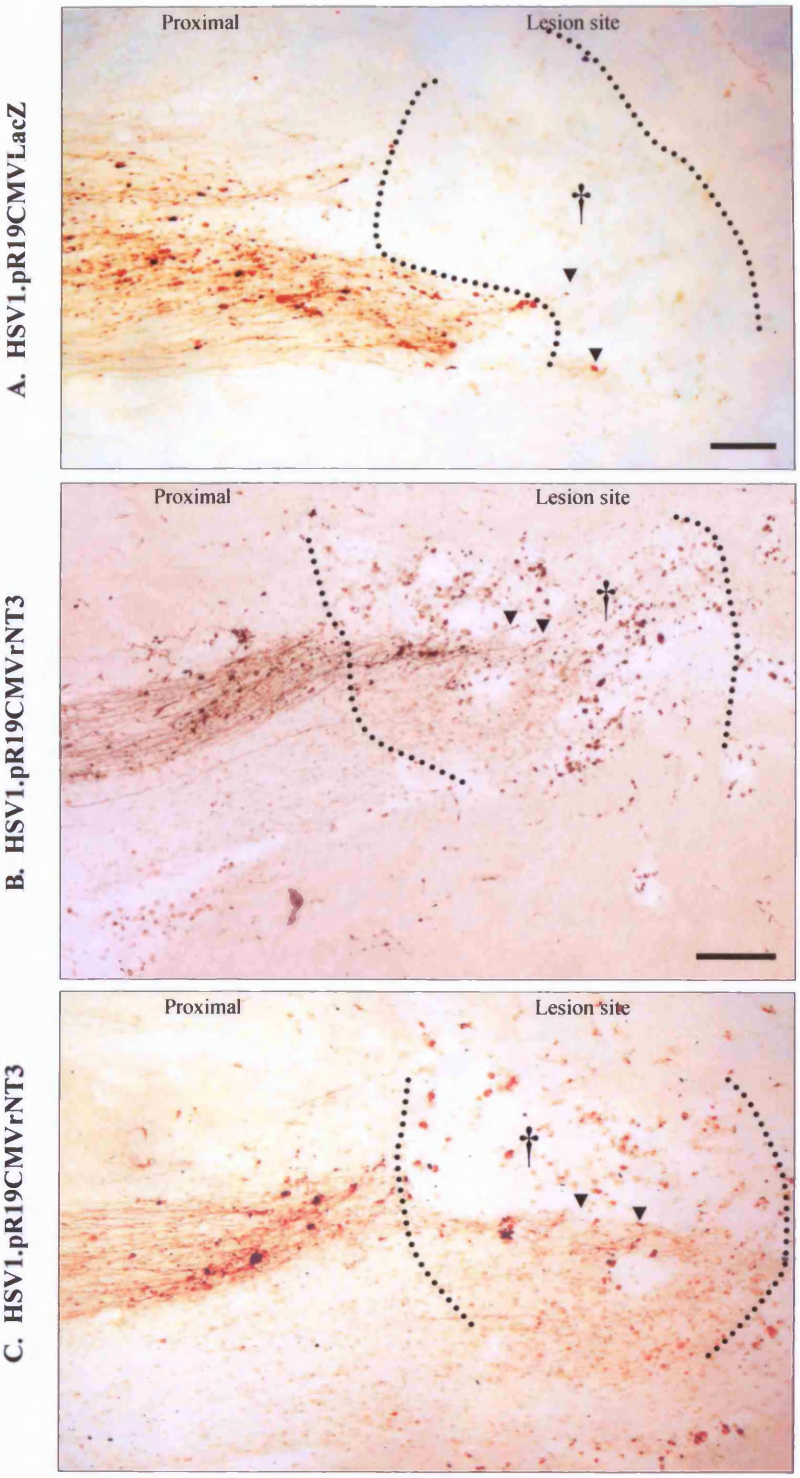
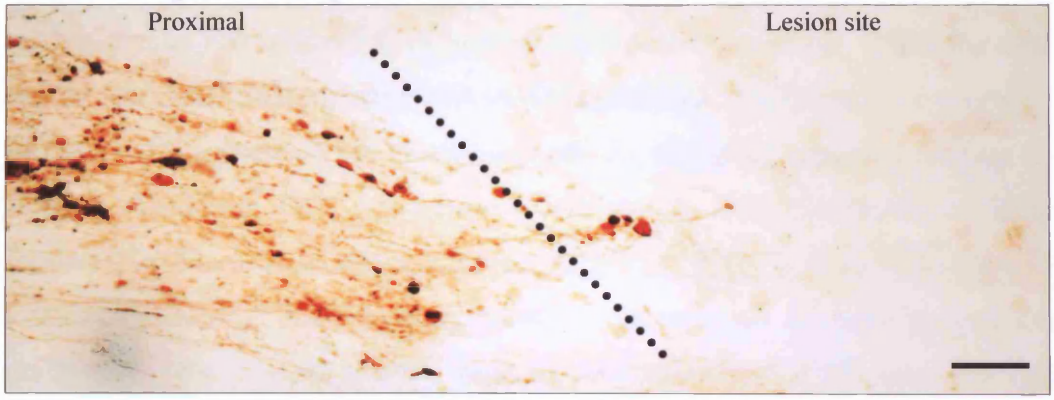

A. HSV1.pR19CMVLacZ
Proximal
Lesion site
B. HSV1.pR19CMVrNT3
Proximal
Lesion site
C. HSV1.pR19CMVrNT3
Proximal
Lesion site

Figure 4.3.3-c: E14 transplants & HSV1.pR19CMVrNT3 promote CST axonal regrowth.

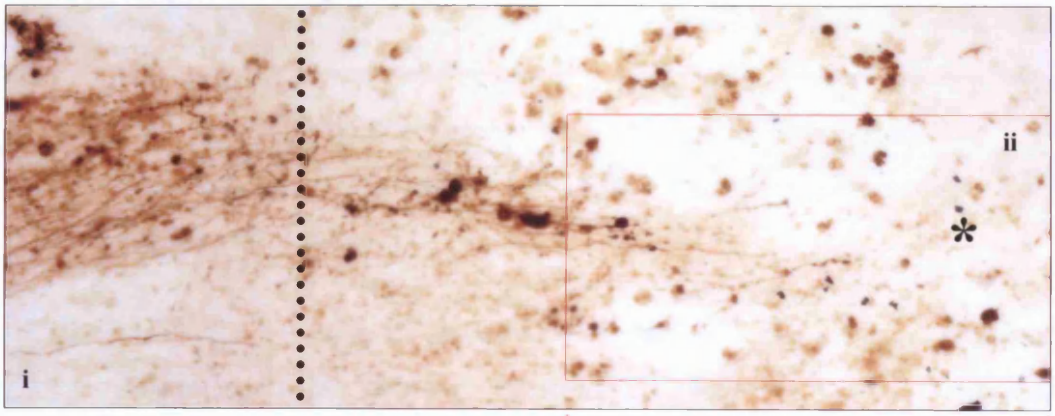
BDA-labelled CST axons regenerating into the lesion site of the spinal cord of animals receiving an HSV1.pR19CMVLacZ/E14 transplant (**A**) or HSV1.pR19rNT3/E14 transplant (**B**) as seen at lower magnification in Figure 4.3.3-b Few axons are seen to sprout across the border of proximal cord and lesion site (dotted line) in the HSV1.pR19CMVLacZ treated animal (**A**). However, many axons sprout into the lesion site in the NT3 vector treated animal (red rectangle in **Bi**, shown at higher magnification in **Bii**) including one (* and arrowed) at 600μm from the proximal boundary (dotted line). (▼regenerating axons).

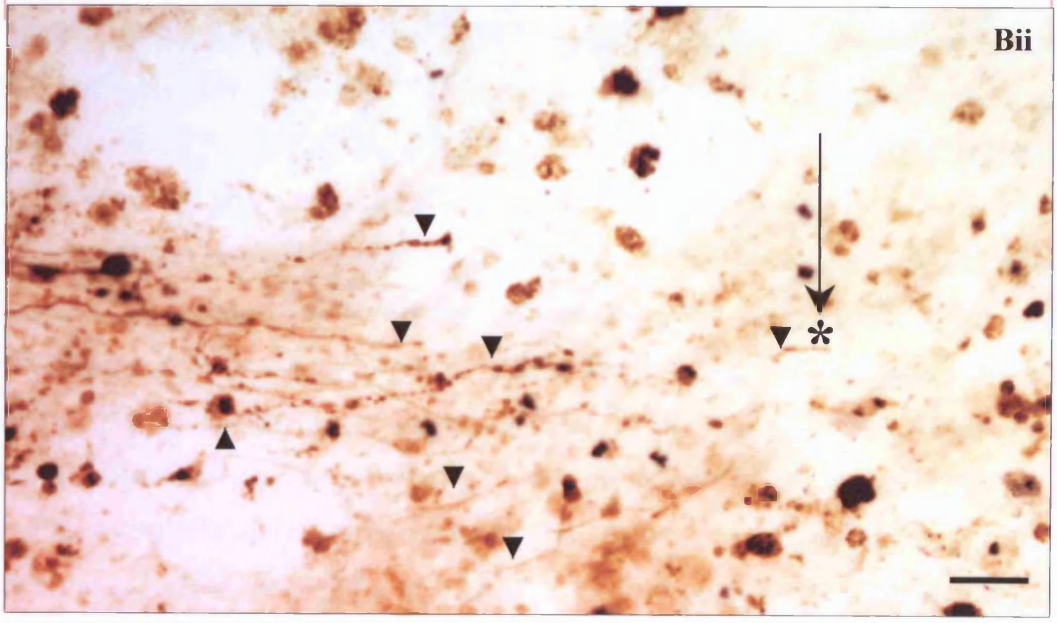
Scale bars: **A**=60μm, applies to **Bi**; **Bii**=30μm.

A. E14 transplant & HSV.pR19CMVLacZ



B. E14 transplant & HSV.pR19CMVrNT3





4.3.4 CST transduction via an HSV1.pR19EF1aWPRE vector

Sole HSV1.pR19CMV mediated expression of rNT3 from neurons surrounding the lesion site was not sufficient to induce a regenerative effect. When the same vector was used in conjunction with an E14 embryonic transplant there was some evidence of regeneration but no axons exited the transplant caudally. Perhaps the lack of significant CST regeneration observed in both paradigms could be attributed to the lack of endogenous production of NT3 from axotomised CST neurons themselves. Endogenously produced factors may be more powerful at augmenting the limited growth capacity of axotomised CST neurons. This approach was not possible due to the fact that HSV1.pR19CMV based vectors turned out to be incapable of sustaining transgene expression in adult CST neurons following either a cortical or spinal cord injection (Chapter 3). However, in order for this type of technology to be realistically applicable in CST regeneration studies, vectors should be capable of directly transducing CST neurons via either of these routes.

As this study was reaching its completion, a new generation of less disabled HSV1 based vectors became available. These vectors designated 1764/ICP27^{+}/ICP4^{-}/RL1^{+}/pR19EF1αWPRE (HSV1.pR19EF1aWPRE), carry the *in*1814 inactivating mutation on VP16 (Ace *et al.*, 1989) and are deleted for the ICP4 trans-activator. The HSV1.pR19EF1aWPRE backbone was based on the well characterised 1764/ICP27^{-}/ICP4^{-}/ICP34.5^{-} genotype (Coffin *et al.*, 1996; Lilley *et al.*, 2001b) and was produced by re-inserting the ICP27 and ICP34.5 genes back into the viral genome via homologous recombination. It should be made clear though that despite its level of disablement this vector is incapable of *in vivo* replication or trans-synaptic spread. It is however easier to propagate in culture and stocks can be grown in higher titres. Preliminary studies using dissociated E14 cortical neurons demonstrated that even though the vector was less disabled, transgene expression was not associated with evidence of cytotoxicity, even when these were infected at an MOI of 5 (personal observations). The rationale behind using this generation of less disabled HSV1 vectors for transducing cortical neurons comes from a previously published study that demonstrated that the cortical pattern of expression following a striatum inoculation appeared to vary

according to the level of vector disablement. Utilising the pR19CMV expression cassette inserted into the minimally disabled and relatively toxic 17^{+}/ICP27^{-} vector (Howard *et al.*, 1998) significant transduction of cortical neurons was observed but only up to 1 week p.i. On the contrary, utilising the more disabled and non-toxic vector 1764/ICP27^{-}/ICP4^{-}, cortical expression was minimal (Lilley *et al.*, 2001b). Therefore, it is very likely that long-term expression in cortical neurons can be achieved by striking the right balance between the level of disablement and toxicity.

The HSV1.pR19EF1aWPRE vector employed the pR19 expression cassette (Lilley *et al.*, 2001b) but two elements of the original construct were altered. Firstly, the CMV promoter was substituted for the human elongation factor I alpha (EF1α) promoter (Kim *et al.*, 1990). The EF1α protein is a key component of a cell's translational machinery as it is part of the elongation factor 1 complex promoting the GTP-dependent binding of aminoacyl-tRNA to the ribosome during peptide elongation. Synthesis of this component of the translational machinery occurs in all cells and is very likely that its promoter will also be active in most cell populations. It was recently reported that EF1α mRNA is present in large amounts in the dendrites of layer V pyramidal neurons in the adult mouse (Huang *et al.*, 2005). It therefore presented a suitable candidate for supporting transgene expression in the same population of neurons. The incorporation of the EF1α promoter was part of a separate study that set out to explore different pR19 expression cassettes, each with a different promoter, for their effectiveness in transducing different neuronal populations *in vivo*. Secondly, the WPRE post-transcriptional regulatory element was inserted at the 3' location of the transgene. WPRE is required for the accumulation of wild type WHV RNAs (Donello *et al.*, 1998) and has previously been shown indirectly to enhance transgene expression in retroviral and lentiviral vector systems, both *in vitro* (Zufferey *et al.*, 1999; Loeb *et al.*, 1999) and *in vivo* (Paterna *et al.*, 2000; Klein *et al.*, 2002; Glover *et al.*, 2003; Hlavaty *et al.*, 2005). The WPRE element is thought to exert this effect by enhancing the nuclear export of mRNAs into the cytoplasm (Zufferey *et al.*, 1999). Therefore it could result in even higher transgene mRNA levels with HSV1 based vector systems as well.

4.3.4.1 Transduction of CST neurons via striatum inoculation

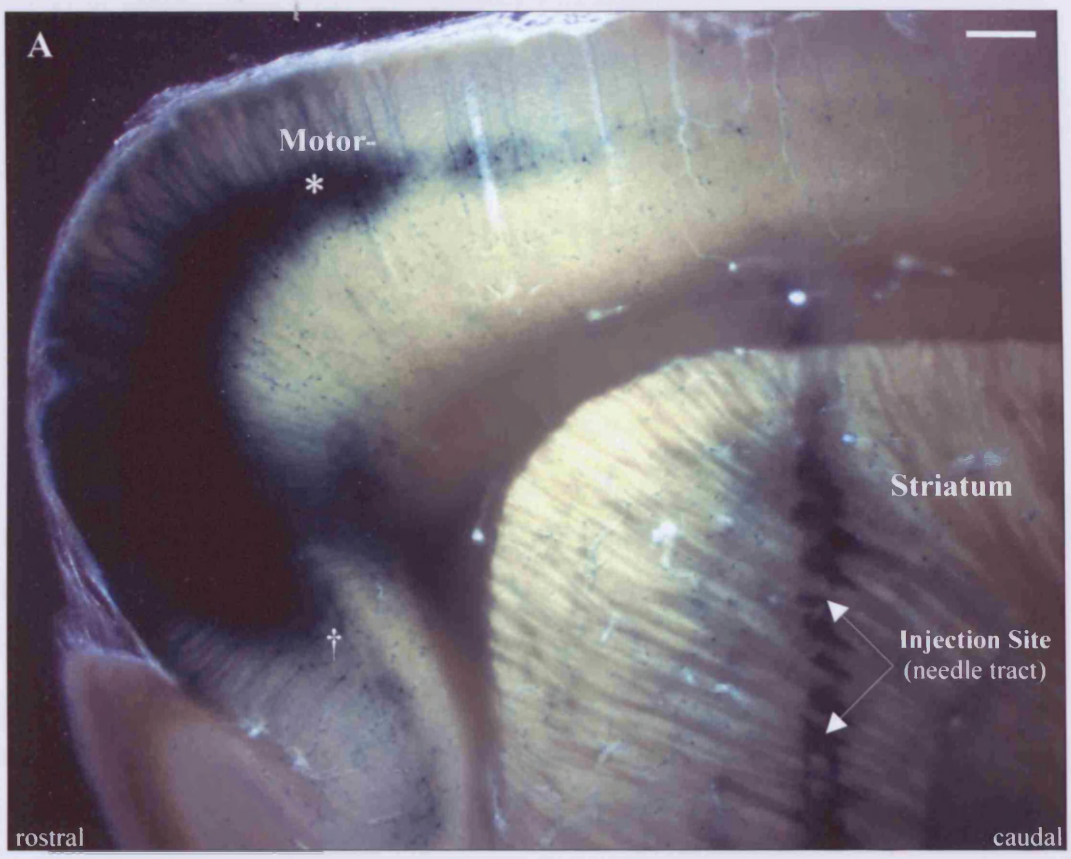
Successful transduction of cortical neurons that project to the spinal cord would assess whether HSV1.pR19EF1aWPRE vectors can sustain long-term transgene expression when administered to the somata of layer V upper motor neurons, following or preceding an acute or chronic lesion within the CST pathway. The transgene, such as NT3, should be endogenously produced and, depending on its properties, be transported throughout the injured axon. This would be ideal for injured spinal cord studies, as the already damaged tissue would be spared any further insult associated with local vector delivery. It would also be beneficial in studies designed to evaluate molecules thought to augment the growth capacity of CST neurons or to counteract any inhibitory influences. Attempts at delivering the vector directly into the cortex were not systematically pursued not only due to time constrains but also because the delivery technique itself would result in some mechanical damage. This is avoided by delivering the vector into the striatum where cortical neurons project thus reducing the possibility of them being lost through injury. $5x10^7$ pfu of HSV1.pR19EF1aWPRE vector (n=3) was inoculated into the striatum of adult rats. Transgene expression was examined on 100μm parasagittal brain sections and at a single time point of one-month post injection.

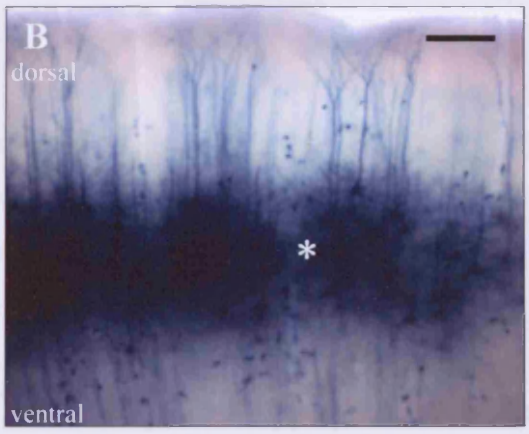
Striatum inoculation of this vector appears to result in three distinct neuronal populations significantly expressing LacZ: the first are striatal neurons around the lesion site, the second are neurons in the primary motor cortex (Figure 4.3.4-a) and the third are substantia nigra neurons (not illustrated). Transduced neurons appeared in clusters with distinct cell bodies, axons and dendritic trees, which in the case of cortical neurons extended from layer V to layer I of the cortex (Figure 4.3.4-a, panel **B**). The results presented here, are part of a preliminary study and do not go as far as confirming that the cortical neurons transduced via this method do indeed project to the spinal cord. Some may be CST neurons but they may also be corticostriatal neurons. To confirm that any transduced pyramidal neurons of layer V of the motor cortex correspond to CST neurons, it was necessary to attempt their direct transduction from within the spinal cord.

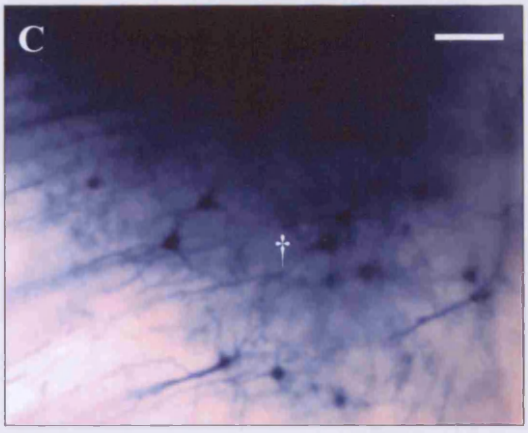
Figure 4.3.4-a: HSV1.pR19EF1aWPRE transduction of cortical neurons.

Low magnification micrograph of a 100μm parasagittal section through rostral cortex and striatum of a rat that received a vector injection into the striatum and survived for 1 month. Large numbers of HSV1.pR19EF1aWPRE transduced, LacZ positive neurons almost fill layer V of the entire rostral tip of the cortex. Groups of these transduced neurons (* and †) are shown at higher magnification in **B** and **C** where their typical pyramidal cell morphology is obvious. The majority of these transduced neurons are likely to be corticostriate neurons. Which of them project to the spinal cord is not established. Very few neurons were transduced close to the track of the injection pipette in the striatum (*). Most soma-sized blue dots in the striatum (and some of those in the deeper layers of cortex) are LacZ positive peri-vascular macrophages.

Scale bars: **A**=200μm; **B**=100μm; **C**=75μm.


A
Motor-
*
†
Striatum
Injection Site
(needle tract)
rostral
caudal


B
dorsal
*
ventral


C
†

4.3.4.2 Transduction of CST neurons via spinal cord inoculation

This experiment was designed to assess whether HSV1.pR19EF1aWPRE vectors can directly transduce cortical motor neurons. This would enable us to study the effects of vector application subsequently to a spinal cord injury, and evaluate the transgene of interest for its effectiveness in aiding regeneration in either the acute or chronic injury models. $5x10^7$ pfu of HSV1.pR19EF1aWPRE vector was inoculated into the dorsal column at the C3 level of adult rat spinal cords (n=5). Transgene expression was examined on 40 μm longitudinal spinal cord sections and at a single time point of 1-month post injection.

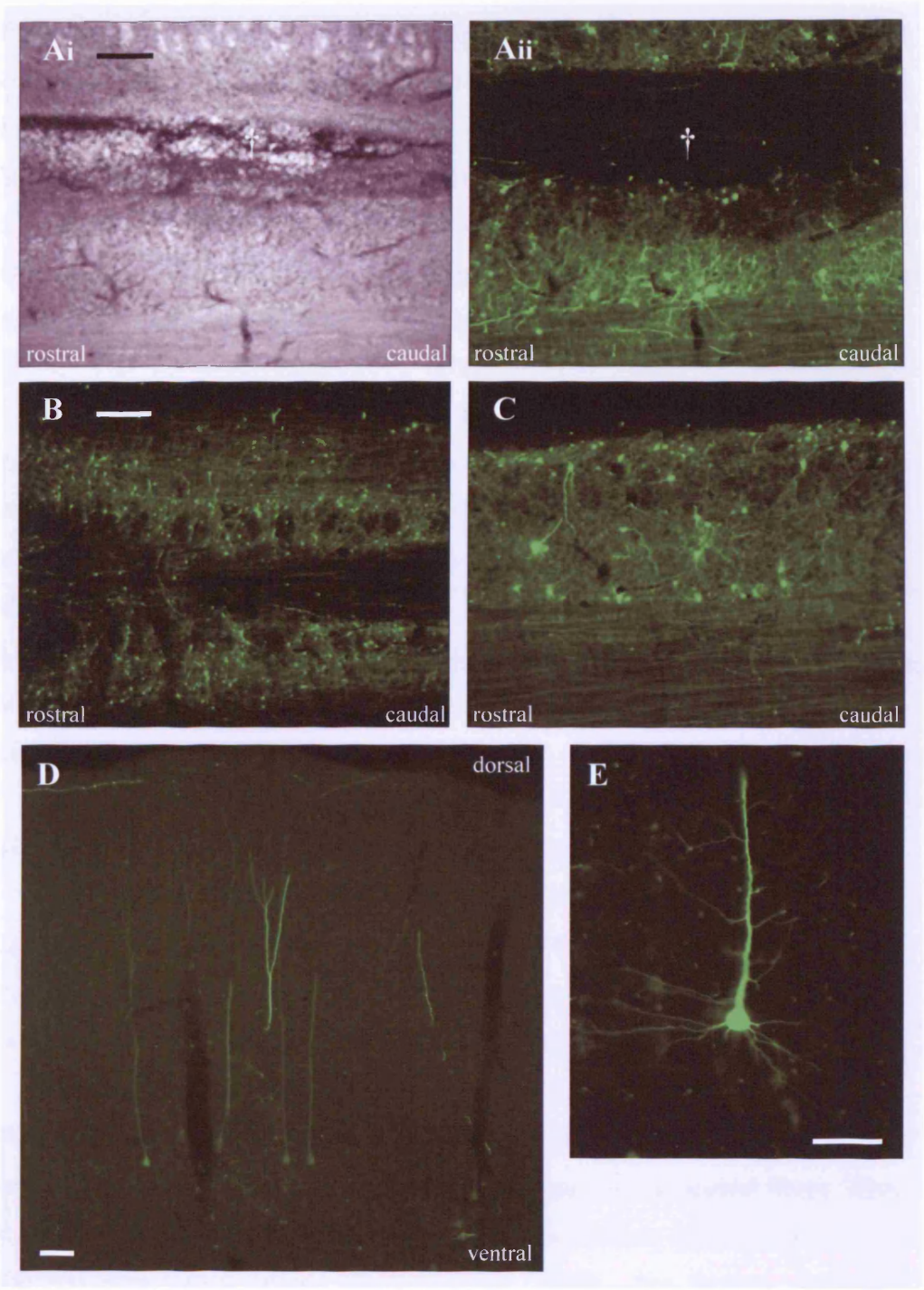
Expression of GFP is strong at that time point in all 5 animals (Figure 4.3.4-b). The marker protein was found not only immediately near the injection site (panels **Ai & Aii**), but also proximal (panel **B**) and distal (panel **C**) to it. GFP labelling was present in large numbers of neuronal cell bodies and axons throughout the entire spinal cord examined (approximately from C5 to medulla). Analysis of 40 μm coronal brain sections of motor cortex demonstrate that this vector allows the transduction of CST neurons following a direct dorsal column inoculation (panels **D & E**). Approximately 100 sections through the motor cortex of each animal were produced. These appeared uniform in terms of the numbers of transduced CST neurons present, with 20 to 40 found bilaterally in each 40 μm coronal section. The somata, characteristic apical dendrites and axons of some CST neurons appeared strongly labelled with GFP (panel **F**). A rough estimate of the numbers of CST cell bodies transduced in a single animal would be in the range of 200-400. Not all transduced neurons appear to express GFP with the same intensity. This may be a reflection of differences in the copy number found within each transduced cell. Transduced neurons were found throughout the brain in regions containing remote projections to the spinal cord, medulla, pons and midbrain. Even though the cortical neurons transduced via a single spinal cord injection form a small proportion of the 79,000 layer V cortical neurons that form the pyramidal tract and project to the C3 level (Gorgels *et al.*, 1989), this approach is promising for regeneration studies targeting the CST. Optimisation of the injection technique could lead to the transduction of more upper motor neuron somata.

Figure 4.3.4-b: HSV1.pR19EF1aWPRE mediated expression in CST pyramidal neurons.

Efficient expression of GFP by spinal cord neurons (**A** to **C**) and cerebral cortex (**D**, **E**) following vector delivered via a single injection into the dorsal columns at the C3 level, 1 month previously. Large numbers of strongly transduced neurons fill the gray matter and many GFP positive axons line the white columns. Expression is strong in somata and axons close to the lesion site (†), which is revealed in the same section taken under dark-field illumination (**Ai**) and under fluorescence microscopy (**Aii**). Similar efficient transduction was observed several millimetres proximal (**B**) and distal (**C**) to the injection site. Note that the majority of transduced cells appear neuronal, not glial and that in **A**, the centre of the injection site (†) does not appear to be infiltrated with autofluorescent inflammatory cells or to be damaged by cavitations.

Transduced GFP positive corticospinal pyramidal neurons (**D**, **E**) in the motor cortex of the rat receiving HSV1.GFP vector in the dorsal columns of the spinal cord shown in **A** to **C**. Approximately 6 moderately to weakly labelled CST neurons are shown in **D** and a strongly GFP positive pyramidal neuron, filled with the virally delivered transgene, is shown at higher magnification in **E** where the thick apical dendrite, basal dendrites and axon are clearly seen.

Scale bars: **Ai**=100μm, applies to **Aii** and **C**; **B**=200μm; **D**=100μm; **E**=50μm.


Ai
rostral
caudal
Aii
rostral
caudal
B
rostral
caudal
C
rostral
caudal
D
dorsal
ventral
E

4.4 Discussion

The chronically injured CST of adult rats has been the focus of the regenerative attempts reported in this Chapter that employ the use of neurotrophins delivered by means of a highly disabled viral vector. This study was more qualitative in the sense that it was designed to explore the ability of this generation of vectors to induce an effect in the injured spinal cord model and in the transected CST model in particular. The vector backbone chosen for these experiments has previously been shown to be non-toxic to the injured spinal cord while at the same time capable of supporting transgene expression for at least one month (Lilley *et al.*, 2001b). The HSV1.pR19CMVrNT3 construct was shown to produce rNT3 protein both *in vitro*, in transduced non-complementing cells and *in vivo*, in the adult rat spinal cord. In vector transduced spinal cords the amount of total NT3 protein was found to be ≈500 pg/mg of total protein, one month following vector inoculation. Importantly, it was shown that the produced protein was capable of inducing a biological effect on embryonic DRG explants. Following confirmation of the bioactivity of vector encoded rNT3, its ability to promote regeneration in the chronically injured CST either alone or in conjunction with an embryonic spinal cord transplant was studied.

Sole HSV1.pR19CMV vector-mediated rNT3 delivery, both proximal and distal to the lesion site, appears to have minimal anatomical effects on inducing regeneration following a cervical level, chronic CST lesion. There was some evidence of a reduced number of end-bulbs, marginally enhanced sprouting proximally to the lesion site and very few fibres present within the lesion site *per se*. As the model utilised in this study involves the bilateral lesioning of the dorsal columns and the unilateral lesioning of the ventral funiculus and intervening grey matter, it is unlikely that the sprouting observed was due to spared fibres. Also the appearance of proximal axons adopting a random course, is in agreement with published regeneration criteria (Steward *et al.*, 2003). The findings described here re-enforce reports that sole administration of the NT3 is inadequate in promoting significant regeneration of CST fibres. A common denominator in reports of any regenerative success in the adult CNS with sole NT3 administration is that the amount of recombinant protein used was biologically

unreasonable. For example, following an intrathecal infusion of 12 μg/day/4weeks of recombinant human NT3, dorsal column sensory axons, demonstrated extensive sprouting proximal to the lesion site and regeneration through the epicentre of the lesion (Bradbury *et al.*, 1999). Even in this case though the path that growing fibres followed was random and no meaningful growth was observed. Intrathecal treatment of lesioned animals with 12 μg/day of NT3 for 4 weeks resulted in the up-regulation of the low affinity $p75^{NTR}$ receptor by glial cells within and around the lesion site. It has been suggested that this may alter the availability of NT3 to injured axons themselves (King *et al.*, 2000). Also, NT3 has been shown to induce BDNF dependency of axotomised CST neurons by the simultaneous activation of TrkC and $p75^{NTR}$ (Giehl *et al.*, 2001). Taken together these findings could suggest that, in cases where highly concentrated infusions of NT3 are administered into the injured CST, a regenerative response may be due to the ectopic regeneration of sensory afferents and may not be a true reflection of an NT3 elicited biological effect (Ramer *et al.*, 2002; Hagg *et al.*, 2005). In the experiments described here the amount of rNT3 present in rat spinal cords via HSV1.pR19CMVrNT3 vector mediated transduction was calculated as a function of pg/ml of total protein. It would have been better to analyse the amounts of NT3 present *in vivo* as a total amount of rNT3 present per grams of spinal cord. Future experiments should examine whether an NT3 gradient is produced at set distances around the lesion site (e.g. 1mm). This would then be easier to correlate to the physical findings of this study.

Combining vector-mediated rNT3 administration with the grafting of an E14 spinal cord transplant does result in increased regeneration probably due to augmenting the natural capacity of axotomised CST neurons to regenerate. When vector-mediated administration of rNT3 is accompanied by an E14 spinal cord transplant, regeneration of chronically axotomised CST axons was somewhat enhanced compared to control animals. There was increased proximal sprouting with many more CST fibres found within the lesion site. The longest regenerating axon was found ≈600μm from the host/transplant border but no CST axons were found to exit the transplant distally. On the contrary, animals that received an

embryonic transplant without an injection of rNT3 expressing vector demonstrated some proximal sprouting but minimal axonal elongation. Again this finding is in agreement with reports that have attempted to promote regeneration via similar routes (Bregman *et al.*, 1997b; Bregman *et al.*, 1998).

Overall it becomes apparent throughout this study that even though trying to induce regeneration following a chronic CST bilateral lesion may be the ultimate test for a potential regenerative tool, with hindsight, it was perhaps too ambitious. After all, the purpose of this study was primarily to assess the capabilities of this vector system in inducing an effect. Even though the obtained results did not demonstrate CST regeneration of the envisaged and perhaps unrealistic scale, efforts should have been focused in evaluating the extent of the achieved regeneration. In terms of evaluating the ability of this gene therapy system to induce a regenerative effect it may have been more productive to deliver the vector prior to inflicting the injury. This would have allowed CST somata, as well as other neuronal populations within the vicinity of the injection site, to already be producing the transgene. This means that the potentially therapeutic transgene would have been present in significant amounts as soon as the lesion was inflicted. This may have augmented the regenerative capacity of the injured CST neurons (Cai *et al.*, 1999) since priming neurons with neurotrophins enables them to resist the inhibitory cues present within a lesion environment via a rise in intracellular cAMP levels (Cai *et al.*, 2002; Spencer and Filbin, 2004). In any case, after four weeks of transplant and vector treatment it would be expected that regenerating CST axons would be growing through the lesion site and perhaps even traverse the transplant (Steward *et al.*, 2003). Even though the exact number of fibres extending into the transplant was regrettably not established in every section and for every animal, an approximate count of the number of axons regenerating into the lesion site in the rNT3 treated animal shown in Figure 4.3.3-b (panel **B**), revealed 15 axons compared with just 3 located in the LacZ treated animal shown in panel **A**. Thus, the regenerative response achieved, indicates that vector-delivered rNT3 is capable of producing some regeneration when used in conjunction with an E14 spinal cord graft. This is encouraging if one takes into account that Bregman *et al.* (1997) used a 1mg/ml concentration of rNT3

compared to the estimated ≈500 pg/mg delivered by this vector system. It is possible then that in the presence of an embryonic transplant, excessive amounts of the neurotrophin may not be a requirement. Alternatively, delivering NT3 via gel foam could be very inefficient, leaving a lot of the protein unutilised. Specifically, in the case of the Bregman *et al.* (1997) paradigm a piece of gel foam soaked with 1 mg/ml of NT3 was implanted dorsally to the transplant itself (Bregman *et al.*, 1997b; Bregman *et al.*, 2002). Even though there are no data regarding the bioavailability of gel foam delivered NT3, it is not reasonable to suggest that this approach allows for only a short treatment span. Studying the bioavailability of recombinant NT3 released from a fibrin gel showed that 95% of the protein was released within 3 days (Taylor *et al.*, 2004). In addition, with NT3 having a half-life of just 1.28 min (Poduslo and Curran, 1996), it quickly looses its bioactivity as it succumbs to inevitable inactivation via non-specific proteolytic cleavage. Thus, the effect observed in the Bregman *et al.* (1997) paradigm could have been induced by a very small fraction of the total NT3 delivered by the gel foam. On the contrary, vector mediated delivery achieves stable expression levels of biologically reasonable amounts of rNT3 that are maintained for at least a month post injection, giving a more realistic idea of the regenerative potential of axotomised CST fibres.

Both host and donor animals were from the Lewis strain of inbred rats. Unlike the more expensive Fischer 344 rats, Lewis rats are not a pure inbred strain (Festing and Bender, 1984). Therefore, all the animals included in this study were treated with a daily dose of Cyclosporin A (CsA) at 2 mg/Kg, both prior and subsequent to transplant/vector administration in an effort to reduce the possibility of the transplant being rejected. The use of CsA in regenerative experiments is quite common despite well documented reports that it promotes neuroprotection by limiting the extent of demyelination and neuronal cell death that ensues an injury resulting in a better motor outcome (Diaz-Ruiz *et al.*, 1999; Ibarra *et al.*, 2003; Okonkwo *et al.*, 2003; Diaz-Ruiz *et al.*, 2005). Even though the amount utilised here is within the range that is reported to have such an effect (Ibarra *et al.*, 2003), control animals could easily be distinguished from rNT3 treated animals in terms of regeneration. This could be due to the fact that CsA was administered

via the somewhat variable oral route as opposed to the intraperitoneal, intravenous or intrathecal route followed in most documented cases. With hindsight, it would perhaps have been more suitable to utilise the Fischer 344 strain for this experiment in order to eliminate this potentially confounding factor. Previously in our laboratory the use of cyclosporin failed to show a positive impact on the length of transgene expression in DRGs transduced via a sciatic nerve or footpad injection with virus 1764/pR20.5/UL43 (work carried out by Dr R.H. Branson, not published). The same pattern has been observed by other laboratories that showed that cyclosporin treatment failed to enhance the amount of vial genome present in trigeminal ganglia (Halford and Schaffer, 2000). Similarly, another group showed that using cyclosporin does not promote an increase in transgene levels in hypothalamic neurons transduced with an adenoviral vector (Geddes *et al.*, 1996). However, the transgene in this case was LacZ and as discussed in Chapter 3 histological methods of detection may not be accurate enough to detect minor differences. There is one report however that reports some beneficial effect in the length of LacZ expression with the use of an HSV1 vector deleted for ICP4, inactivated for VP16 and vhs, but this utilised the hamster model of viral latency in sympathetic neurons (Mabon *et al.*, 1999).

All the experiments described in this Chapter were based on the chronic model of CST lesion in which treatment with vector or transplant and vector occurred after at least four weeks following the dorsal column lesion. Delaying treatment with embryonic transplants and NT3 by two to four weeks was shown to promote regeneration and some functional recovery in the injured CST (Coumans *et al.*, 2001). However, delaying treatment for six (Shumsky *et al.*, 2003) or eight (von Meyenburg *et al.*, 1998) weeks post lesion results in only minimal recovery of function. The time point selected here is therefore within the window of opportunity for intervention as far as the CST is concerned while at the same time the inhibitory environment of the lesion site are also in place (Shibayama *et al.*, 1998). The identification of the lesion site in this set of experiments was performed by phase contrast and dark-field microscopy on longitudinal sections. The location of the injury was pinpointed as being the opaque tissue in between the rostral and caudal parts of the spinal cord. Even though in the majority of

animals establishing the boundaries of the lesion site using microscopy of this kind was clear-cut, additional analysis should have been employed so that results between animals were evaluated on a common basis. For example, the staining of alternate sections for the expression of GFAP would label the astrocytic barrier formed at sites of injury and it would thus allow a clear demarcation of the lesion site. Alternatively, dissection of tissue blocks that include the lesion site (e.g. 3 mm caudal/3 mm rostral to the lesion site) and subsequent weighting prior to camera Lucida reconstruction of Nissl-stained sections would ensure some degree of standardisation between samples. Any effect on the actual number of end-bulbs or the extent of their retraction should also have been better characterised with statistical analysis supporting the histological data shown here and providing a more accurate picture. In addition, since the levels of regeneration obtained were so limited, some immunohistochemistry should have been carried out in order to ascertain the extent to which vector administration may have altered the post-treatment expression profiles of growth associated proteins such as GAP43. A technical aspect that may have had a negative impact on these studies was the potential inconsistency in viral pfu administered, which in turn resulted in an unpredictability of rNT3 levels subsequently secreted within the inoculated spinal cord. At the time this study was performed there was a distinct lack of NT3 specific antibodies that were sensitive enough to detect it *in vivo*. Perhaps to compensate for this unavoidable variability, the levels of rNT3 expression should have been established by immuno-histochemistry with respect to the vector injection site. This would have also provided valuable data on the relationship between proximity of vector inoculation and transgene expression with relation to any regenerative effect on axotomised CST fibres. Sampling a vector-injected spinal cord at regular intervals proximally, within and distally to the lesion site would provide more information regarding the expression patterns of the transgene as well as the extent of diffusion of the secreted protein itself. Correlating this data with different inoculation sites would perhaps provide a better model of vector administration for this type of experiment.

The potential of being able to deliver transgenes to CST neurons via either of these routes has been the focus of other vector systems with varied degrees of

success. A canine serotype-2 adenoviral vector (CAV-2) delivered into the striatum does result in the retrograde transduction of cortical neurons. Even though the study is not clear as to the time points examined the authors report $\approx 10^4$ cortical neurons transduced following a $2x10^9$ pfu striatum inoculation (Soudais *et al.*, 2001). Another study employing a serotype-5 rAAV vector (rAAV5), led to transduction of only three layer V neurons, ten days following inoculation of $6x10^6$ pfu of vector into the frontal cortex of adult rats (Tamamaki *et al.*, 2000). A similar approach whereby $1x10^6$ pfu of a disabled CMV vector were delivered into the striatum, yielded very limited and short-lived transduction of cortical neurons 3 days p.i. (van Den Pol *et al.*, 1999). One study that does report considerable cortical transduction is that conducted by Sandler and colleagues (2002) who used $2x10^5$ pfu of an HSV1 amplicon vector to retrogradely transduce clusters of layer V pyramidal neurons via a single striatum injection. The transgene was visible from 18 hours to at least five weeks post-injection, but no data were presented regarding the exact number of transduced neurons (Sandler *et al.*, 2002). Hermens *et al.* (1997) infused $5x10^7$ pfu of an adenoviral vector in the corpus callosum of adult rats and successfully transduced pyramidal neurons including some layer V neurons. Expression was noted at 4 days p.i. (Hermens *et al.*, 1997). Neither of the above studies provides any information in relation to the proportion of transduced cortical neurons that form the CST tract. Following the spinal cord route of administration, Burger *et al.* (2004) injected $2x10^{10}$ pfu of serotype recombinant AAV type 2 pseudotyped for serotype 5 (rAAV2/5) vector into the C4 spinal cord level. In this case some cortical labelling was present at one month p.i but the report is too vague in order to be able to draw any conclusions regarding its effectiveness (a single GFP positive neuron is shown) (Burger *et al.*, 2004).

SUMMARY

Vector-mediated transduction of rNT3 alone proved insufficient to promote regeneration in the chronically injured CST. It did however appear to augment the regenerative capacity of CST in the presence of E14 spinal cord transplants placed within the lesion. The findings presented in this chapter could be interpreted to support the notion that, given the highly inhibitory nature of the

injured CNS, it is unlikely that a single approach could lead to functionally successful regeneration (Bunge, 2001). Delivering biologically achievable amounts of neurotrophins, as is possible with the vectors employed here, may need to be coupled with factors that counteract the inhibitory molecules found in myelin and in the injured CNS milieu in general. The vectors used in the regeneration approaches presented here as well as the new generation of less disabled constructs briefly explored in section 4.3.4.2 (page 239) present a powerful tool from which to explore these interactions.

CHAPTER 5

EFFECTS OF HSV1 VECTOR-MEDIATED DELIVERY OF BDNF OR CNTF TO THE INJURED RUBROSPINAL TRACT

5.1 Introduction

The regenerative attempts outlined in this chapter are focussed on inducing rubrospinal tract neurons to regenerate their axons following a unilateral lesion. Like some other long descending tracts in the CNS, adult red nucleus neurons in the caudal magnocellular portion of the red nucleus (MPRN) display significant and progressive atrophy following axotomy resulting from a spinal cord lesion, but do not die (Prendergast and Stelzner, 1976; Barron *et al.*, 1989; McBride *et al.*, 1990). This atrophy is progressive and even though the expression of molecules such as neuronal Tα-1 tubulin are transiently up-regulated after axotomy, there is a gradual decline in regeneration-associated gene (RAG) expression, including GAP43 and other cytoskeletal proteins (Barron *et al.*, 1989; Tetzlaff *et al.*, 1991). The sharp decline in RAG expression is accompanied with the onset of severe atrophy that reaches its peak around 2 weeks following a lower cervical or upper thoracic lesion (Egan *et al.*, 1977; Tetzlaff *et al.*, 1994). The present study was based on the fact that neurotrophin delivery has been used to minimise (Kobayashi *et al.*, 1997) and even overcome (Ruitenberg *et al.*, 2004) post-axotomy neuronal atrophy (Kwon *et al.*, 2002). BDNF administration to red nucleus neurons has been shown to stimulate the expression of genes associated with regeneration and boost the limited ability of axotomised rubrospinal tract fibres to extend their axons into a peripheral nerve graft (Kobayashi *et al.*, 1997; Kwon *et al.*, 2002). Similarly, CNTF has the potential to enhance recovery of axotomised rubrospinal tract neurons (Ye *et al.*, 2004). Delivery of neurotrophins by engineered fibroblasts (Tobias *et al.*, 2003), neural progenitors (Xiao *et al.*, 2005), foetal transplants (Mori *et al.*, 1997; Bregman *et al.*, 1998) as well as viral vector based systems other than HSV1 (Koda *et al.*, 2004; Ruitenberg *et al.*, 2004), have been shown to induce a beneficial response of varying extent. This chapter examines whether delivering neurotrophic factors to the injured rubrospinal tract by utilising highly disabled HSV1 vectors is capable of limiting the extent of red nucleus atrophy and promoting axonal elongation.

Firstly, the ability of a highly disabled HSV1 vector (HSV1pR19CMV) to transduce red nucleus neurons 4 weeks after a lesion of the lower cervical unilateral rubrospinal tract lesion was examined. Once it was confirmed that this

vector backbone was suitable for transgene delivery via this route, two separate, neurotrophic factor expressing vectors were constructed: HSV1pR19CMVmCNTF, expressing a secretable form of the murine CNTF (mCNTF) and HSV1pR19CMVrBDNF vector expressing rat BDNF (rBDNF). Both of these vectors were used either alone or in combination to promote rubrospinal tract axonal regeneration following direct spinal cord delivery. Regeneration was assessed by analysing anterogradely labelled rubrospinal tract axons within sections of spinal cord. Additionally, vector HSV1.pR19CMVrBDNF was used to explore the possibility of rescuing red nucleus neurons from post-traumatic atrophy. One month following vector inoculation and two months post-axotomy, the size of red nucleus neuronal cell bodies was measured and the extent of regeneration with and without treatment was examined.

5.1.1 Anatomy of the rubrospinal tract

The rubrospinal tract is a white matter tract that originates from the red nucleus, which is located in the midbrain just dorsal to the substantia nigra at the level of the superior colliculus. The red nucleus or "ruber", so-called due to the high iron content and extensive vascularisation that gives it its characteristic reddish colour, can be divided into two regions: pars-magnocellularis and pars-parvocellularis. Rubrospinal neuron somata are not distributed uniformly throughout the red nucleus. In rodents, the cell bodies of rubrospinal neurons are larger and are found at a higher density within the magnocellular rather than parvocellular parts of the red nucleus (Shieh *et al.*, 1983; Daniel *et al.*, 1987; Tracey, 1994). There appears to be a degree of somatotopic organisation in the rubrospinal projections from the red nucleus, with its ventro-lateral parts projecting mainly to the lumbar and the dorso-medial parts projecting to the cervical segments of the spinal cord respectively. This division is not absolute and some red nucleus neurons apparently project to both areas of the rat spinal cord (Antal *et al.*, 1992; Tracey, 1994).

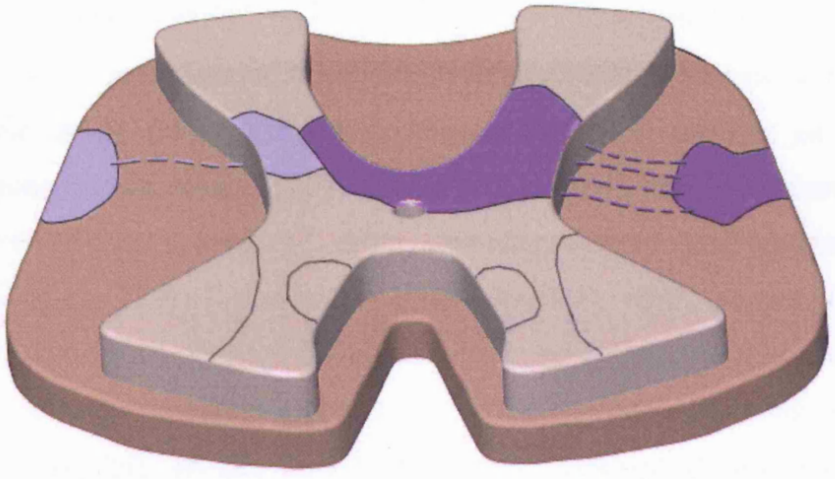


Figure 5.1.1-a: Projections of the rubrospinal tract in the rat.

The rubrospinal tract is confined to a small region of the lateral white column in the spinal cord. Here the contralateral projection (dark purple) lies with a small ipsilateral projection (light purple). Axons of the tract terminate in the ventral half of the dorsal horns. The rubrospinal tract displays somatotopic arrangement (Tracey, 1994; Joosten, 1997).

Rubrospinal axons decussate just caudal to the red nucleus, travel through the brain stem and enter the spinal cord where they travel in the lateral funiculus close to the lateral corticospinal tract. Within the spinal cord, rubrospinal processes cross from the lateral funiculus to the ventral horn to terminate on interneurons, which in turn synapse on lower α-motor neurons (Figure 5.1.1-a). The rubrospinal tract is thought to work with the corticospinal tract to co-ordinate fine motor movement.

5.1.2 Response of red nucleus neurons to axotomising lesions

In line with the behaviour of other neuronal populations intrinsic to the CNS, axotomised adult rubrospinal tract neurons are not capable of unaided regeneration (Barron *et al.*, 1989). Even though rubrospinal tract neurons have been shown to be capable of some sprouting (Houle and Ziegler, 1994; Kobayashi *et al.*, 1997; Kwon *et al.*, 2002) especially when compared to other CNS intrinsic neurons, they too fail to regenerate, becoming severely atrophied in the weeks following axotomy (Egan *et al.*, 1977; Barron *et al.*, 1989; Barron *et al.*, 1990; Tetzlaff *et al.*, 1991). However, rubrospinal neurons, unlike corticospinal neurons, readily regenerate their axons into nerve grafts placed in the cervical spinal cord (Richardson *et al.*, 1982; Richardson *et al.*, 1984).

5.1.2.1 Morphological changes following rubrospinal tract axotomy

In newborn rats, lesions that injure the rubrospinal tract result in profound neuronal cell death in the red nucleus (Prendergast and Stelzner, 1976; Bregman and Reier, 1986). Early experiments addressing the response of adult red nucleus neurons to axotomy report an average neuronal loss of ≈40% in the subsequent two to eight weeks following a lateral funiculus lesion at the upper cervical level (Goshgarian *et al.*, 1983; Feringa *et al.*, 1988; Mori *et al.*, 1997). However it is now widely accepted that adult magnocellular neurons of the red nucleus undergo severe atrophy following either a cervical or thoracic level lesion but with only limited cell death (Egan *et al.*, 1977; Barron *et al.*, 1989; Tetzlaff *et al.*, 1991). The extent of somal shrinkage that follows an upper cervical rubrospinal tract lesion ranges from ≈25% (Novikova *et al.*, 2000) to ≈40% (Mori *et al.*, 1997;

Murray *et al.*, 2002) reduction in cell body size in surviving neurons at 2 weeks and 2-4 months respectively. It is important to emphasise that there has long been a lot of disagreement between research groups as to the extent of atrophy and the suitability of the methods used to measure it (Tetzlaff *et al.*, 1994; Kobayashi *et al.*, 1997; Kwon *et al.*, 2002). Experimental differences between the different studies, in terms of where the axotomising lesion is localised or which part of the red nucleus is assessed for atrophy, can also significantly affect the reported mean somal areas. For example 2% of the magnocellular red nucleus neurons are giant cells (Mori *et al.*, 1997). Mori and colleagues report that red nucleus neurons become atrophic in a non-uniform manner. The majority of neurons that undergo the more profound atrophy following an axotomising lesion belong to that 2% and are the so-called "giant neurons". They become so severely atrophied that they are reported as being almost absent by the 8th month post lesioning (Mori *et al.*, 1997). Understandably, atrophic changes will be more profound in this class of neurons and even though they only represent a 2% of the population, counting studies that exclude them will report different findings to those that include them in any statistical analysis. Several groups acknowledge the fact that they may have overestimated the extent of neuronal death due to the inability to detect severely atrophied red nucleus cell bodies (Tetzlaff *et al.*, 1994; Mori *et al.*, 1997). This is an important factor that was taken into consideration during the design of this study and is further examined in section 5.4 (page 312). Nevertheless some neuronal death does occur in rubrospinal tract axotomy but its extent declines with increasing distance of the lesion from the red nucleus somata (Liu *et al.*, 2003). Moreover, recent studies, utilising the latest counting techniques have questioned whether there is any neuronal loss at all within the adult red nucleus following axotomy. Specifically, a closed rubrospinal tract axotomy that preserves BBB integrity, inflicted at the level of the brain stem, results in a ≈57% red nucleus neuronal loss within the first two weeks, with an additional ≈18% reduction in from week two to week 10 following the injury (Liu *et al.*, 2003). This is significantly higher than the ≈7% loss noted following a C2 level axotomy which rises to a modest ≈8% within the same time frame (Wang *et al.*, 2002; Liu *et al.*, 2003). These dissimilar morphological responses are accompanied by molecular differences. For example, neuronal nitric oxide

synthase (nNOs) expression is upregulated more strongly when an rubrospinal tract lesion is located at the brainstem rather than the C2 level while nNOs upregulation is not detected following a T10 level injury (Liu *et al.*, 2004). The increased production of nitric oxide (NO) plays an important part in the significant neuronal loss that follows rubrospinal tract lesions close to the red nucleus somata, since inactivating nNOs leads to a reduced red nucleus neuronal death from ≈55% down to ≈33% by the end of the second week post lesion (Liu *et al.*, 2004).

In addition to changes to the neurons in the axotomised red nucleus, there are also changes to the astrocyte and microglial populations. For example, following axotomy there is an increase in the density of processes of both these glial populations within the red nucleus. It has been suggested that this may affect the ability of red nucleus neurons to regenerate their axons (Tseng *et al.*, 1996a; Tseng *et al.*, 1996b). The extent of the astrocytic and microglial responses that follow a rubrospinal tract lesion also depend on the level of the lesion and may be related to the regenerative failure of red nucleus neurons (Barron *et al.*, 1990; Tseng *et al.*, 1996a; Tseng *et al.*, 1996b). An upper cervical, lateral funiculus lesion does not cause microglial hyperplasia (Barron *et al.*, 1990). Tseng and colleagues (1996a and b) assessed the difference in the microglial and astrocytic response that ensues a cervical versus a lower thoracic rubrospinal tract axotomising lesion. These studies revealed that following either a C1-C4 or a lower thoracic (T10) level rubrospinal tract lesion in young adult rats, there is a relatively modest and biphasic activation of microglia (Tseng *et al.*, 1996a; Novikova *et al.*, 2000) and astrocytes (Tseng *et al.*, 1996b), with both phases being more prominent after a cervical level lesion. The biphasic response is by far weaker than that observed in the case of a peripheral nerve lesion (Barron *et al.*, 1990) and can be divided into an early phase, spanning the first 2-5 days and a late phase between 2-8 weeks post lesioning (Tseng *et al.*, 1996a; Tseng *et al.*, 1996b). Even though no mitotic proliferation occurs, microglial and astrocytic processes become denser following a rubrospinal tract axotomy, which as discussed in Chapter 1, is indicative of their activation. However, the microglial and astrocytic processes do not appear to wrap the red nucleus neuronal somata

(Tseng *et al.*, 1996a; Tseng *et al.*, 1996b) as they do in the case of motor neurons axotomised by a peripheral nerve injury that allows regeneration of their axons (Streit *et al.*, 1988). Tseng and colleagues (1996a) suggested that the lack of microglial hyperplasia could be related to the inability of red nucleus neurons to regenerate (Streit *et al.*, 1988; Barron *et al.*, 1990). This is in line with the lack of such a response in other non-regenerative tracts such as the CST (Leong *et al.*, 1995). Interestingly, following either an axotomising cervical or thoracic lesion the limited microglial and astrocytic response that does follow occurs not only in the contralateral red nucleus as expected but it is equally present in the ipsilateral nucleus (Tseng *et al.*, 1996a). The authors of this finding argue that this may be due to axotomised bilateral projecting red nucleus neurons with axons projecting bilaterally (Huisman *et al.*, 1981). Lesioning of rubrospinal tract axons causes retrograde retraction of neuronal terminals from the lesion site that differs from the progressive degenerative response seen in the case of axotomised CST neurons (Pallini *et al.*, 1988). Chronically injured cervical rubrospinal tract axons begin to undergo retraction soon after lesioning with over 60% of terminals found at 400-500 μm from the lesion site at 1-week post operatively (Houle and Jin, 2001). This retraction is non-progressive, even when examined at 14 weeks postoperatively. Even at such a distant time point 50% of red nucleus retraction bulbs can be found within 500 μm from the lesion site (Houle and Jin, 2001).

5.1.2.2 Molecular changes following rubrospinal tract axotomy

Undoubtedly, as with other neurons that are intrinsic to the CNS, there are various reasons why injured red nucleus neurons do not regenerate their axons. Many molecules including c-Jun, ATF3, GAP43, and Trks charge their expression patterns in response to axotomy. These changes as well as how they impact the regenerative potential of axotomised red nucleus neurons is examined below.

From 7 days following cervical axotomy, magnocellular red nucleus neurons upregulate GAP43 and α1 Tubulin mRNA levels while they down-regulate neurofilament mRNA, as detected by in situ hybridisation (Tetzlaff *et al.*, 1991; Fernandes *et al.*, 1999). However, this is neither a uniform nor a sustained

response with mRNA levels diminishing as atrophy sets in (Tetzlaff *et al.*, 1991). Subsequent to the initial burst of activity, mRNA levels decline to subnormal levels, seven weeks after a C3 level axotomy (Tetzlaff *et al.*, 1991; Fernandes *et al.*, 1999). This upregulation of growth-associated proteins does not occur at all if the rubrospinal tract lesion is carried out at the thoracic level (Fernandes *et al.*, 1999). In this case, red nucleus neurons fail to upregulate GAP43 or Tα1-Tubulin transcription while neurofilament mRNA levels decline following either a cervical or thoracic lesion (Fernandes *et al.*, 1999). Additionally, even though cervical axotomy leads to the upregulation of c-Jun expression in red nucleus neurons, a similar lesion of the thoracic spinal cord fails to do so (Jenkins *et al.*, 1993; Houle *et al.*, 1998). Levels begin to rise within the first 24 hours, reach their peak at 10 days and persist for at least 4 weeks following a C3 level lesion (Jenkins *et al.*, 1993; Houle *et al.*, 1998). Upregulation of the c-Jun transcription factor has been associated with neurons that are capable of at least initiating a regenerative response (Houle *et al.*, 1998; Mason *et al.*, 2003). This difference between cervical and thoracic lesions and their potential in up-regulating growth associated proteins may not translate to an increased ability to regenerate spontaneously but correlates to their ability to regenerate into, but not through, a peripheral nerve, transplanted within the lesion site (Richardson *et al.*, 1980; Richardson *et al.*, 1982; Kwon *et al.*, 2002).

Non-axotomised magnocellular red nucleus neurons have been shown to express NgR (Hunt *et al.*, 2002b) and Nogo-A mRNA (Hunt *et al.*, 2003) as detected by *in situ* hybridisation, albeit weakly. In *ngr-/-* mutant rats, red nucleus neurons are capable of axonal regeneration following a thoracic level spinal cord transection (Kim *et al.*, 2004). The authors argue that this may point to the fact that NgR signalling plays a part in the limited ability of red nucleus neurons to mount a successful regenerative response. Healthy red nucleus neurons are positive for the presence of α7, αV and β1 integrin receptor subunit mRNA (Pinkstaff *et al.*, 1999). Integrins are responsible for interaction of neuronal growth cones both with other cells and ECM components and can therefore define the nature of the neuron's response to injury (Letourneau *et al.*, 1994; Jones, 1996; Condic and Letourneau, 1997). Expression is high in the embryonic stages and early postnatal

period (Letourneau *et al.*, 1994; Jones, 1996; Condic *et al.*, 1999) but ceases in the adult CNS (Jones, 1996). Different subunit combinations are responsible for ligand specificity of the integrin receptor. For example, in DRG neurons following a conditioning lesion, it is only the expression of subunits α7 and β1 that are upregulated, at least *in vitro* but no axonal regeneration occurs in the presence of anti-β1 subunit neutralising antibodies, even in the presence of the α7β1 specific ligand: laminin-1 (Ekstrom *et al.*, 2003). The authors of the above study suggest that this points to the importance of the β1 subunit in axonal elongation, at least in peripheral neurons. Restoration of integrin levels to those found in the early postnatal period of development can induce adult neurons to display axonal regenerative abilities similar to those seen in the embryonic and postnatal stages (Condic, 2001). Following a C3 level rubrospinal tract axotomy in adult animals only subunit β1 is upregulated to levels detectable by *in situ* hybridisation. Even though this may create an abundance of subunit β1, formation of functioning heterodimers is either not possible as the α subunits are not upregulated in the same manner, or subunit β1 interacts with an as yet unidentified α subunit (Plantman *et al.*, 2005). This may be one of the factors contributing to the inability of red nucleus to regenerate their axons.

In addition to the expression patterns described above, the behaviour of another molecule of emerging interest has also been examined with respect to its expression in the axotomised rubrospinal tract. Following a C4 level axotomy, Galectin-1 (Gal-1) mRNA levels decline in red nucleus neurons within seven to fourteen days, as assessed by *in situ* hybridisation (McGraw *et al.*, 2004b). This notion, put forward by McGraw and colleagues, is supported by the fact that the expression of Gal-1, positively correlates with the ability of adult (Fukaya *et al.*, 2003) as well as developing (Regan *et al.*, 1986) neurons to successfully up regenerate their axons. Injured adult spinal motor neurons can significantly up-regulate the production of Gal-1 within 24-hours following facial nerve axotomy that precede the subsequent regenerative response (Akazawa *et al.*, 2004). Moreover, in the adult, application of recombinant human, oxidised version of Gal-1 can potentiate the regenerative response of both sensory and spinal motor neurons (Fukaya *et al.*, 2003).

Magnocellular red nucleus neurons display strong immunoreactivity for TrkC and TrkB, both of which are significantly down regulated over the weeks following a cervical level rubrospinal tract lesion (Kobayashi *et al.*, 1997; Yan *et al.*, 1997; Novikova *et al.*, 2000). Levels begin to decline from 7 days post axotomy and continue to do so over the next two weeks, coinciding with the onset and progression of red nucleus atrophy (Kobayashi *et al.*, 1997). On the contrary, TrkA is absent from uninjured red nucleus neurons and present in very few red nucleus neurons following a C4 level, rubrospinal tract axotomy (Kobayashi *et al.*, 1997; Yan *et al.*, 1997). In addition, $p75^{NTR}$ mRNA expression is undetectable in uninjured red nucleus neurons but appears marginally increased at 7 days but not at 3 weeks post lesion. The expression patterns of the above neurotrophin receptors support the notion that BDNF and NT3 but not NGF treatment has the potential to positively influence the response of axotomised red nucleus neurons (Kobayashi *et al.*, 1997).

Attempts at achieving regeneration in the rubrospinal tract injury model often involve the transplantation of growth permissive substrates and/or removal of scar tissue at the lesion site. This approach is relatively invasive and may result in a secondary axotomy being inflicted, which may in turn lead to an upregulation of growth-associated proteins. Re-lesioning the rubrospinal tract at 28 days after a C3 level axotomising lesion enhances the mRNA levels of GAP-43 and βII-Tubulin in red nucleus neurons and this response in maintained for at least 7 days (Storer and Houle, 2003). The onset of this response appears to be more rapid and proportionally higher than that observed following the acute phase of the primary lesion (3 days). Storer and Houle (2003) suggest that the ability of these neurons to respond to injury is indicative of the fact that chronically injured red nucleus neurons are still able to mount a response to secondary axotomy, at least at the mRNA level. A second axotomy carried out eight weeks after the primary lesion, leads to the upregulation of c-Jun expression, even though this appeared to be silenced four weeks after the primary C3 lesion was inflicted (Houle *et al.*, 1998). Small neurons of the magnocellular portion of the red nucleus appear to be more sensitive to the effects of a second lesion (Houle and Ye, 1999).

5.1.3 Promoting regeneration in the injured rubrospinal tract

As with other tracts of the CNS various methods have been employed to encourage axonal regeneration or to prevent/reverse the atrophy of the axotomised perikarya (Egan *et al.*, 1977; Barron *et al.*, 1989; Tetzlaff *et al.*, 1991). Most of these attempts are centred on the administration of neurotrophic factors, the effects of which are considered in more detail in section 5.1.4. Undoubtedly, the implantation of the end of a peripheral nerve into a lesion site in the lateral funiculus leads to a modest regeneration of rubrospinal tract axons into the graft (Richardson *et al.*, 1982; Houle, 1991). Regeneration of rubrospinal axons into the grafts is more extensive if the graft is implanted into the cervical rather than the thoracic spinal cord (Richardson *et al.*, 1984), presumably because of a more vigorous cell body response (Fernandes *et al.*, 1999). The results may vary depending on the time between injury and grafting. For example, immediate grafting of a peripheral nerve into the lesion site of a C3 level hemisection lesion site appears to be more effective in limiting red nucleus atrophy than the same procedure 3 weeks or more after injury (Richardson *et al.*, 1984; Decherchi and Gauthier, 2000). Similarly, the unaided transplantation of foetal spinal cord into the lesion site of a similar injury is not sufficient to promote any meaningful rubrospinal tract regeneration (Bregman and Reier, 1986; Mori *et al.*, 1997). On the other hand, human adult olfactory neural progenitor cells have been shown to be able to rescue red nucleus neurons from atrophy following a C3-C4 axotomy, possibly because they are capable of endogenously producing significant amounts of neurotrophic factors such as BDNF (Xiao *et al.*, 2005; Xiao *et al.*, 2007). This is in contrast to mouse olfactory ensheathing cells, which failed to produce regeneration of rubrospinal axons in the rat or mouse model (Ramer *et al.*, 2004).

5.1.4 Neurotrophin treatment and red nucleus neuron regeneration

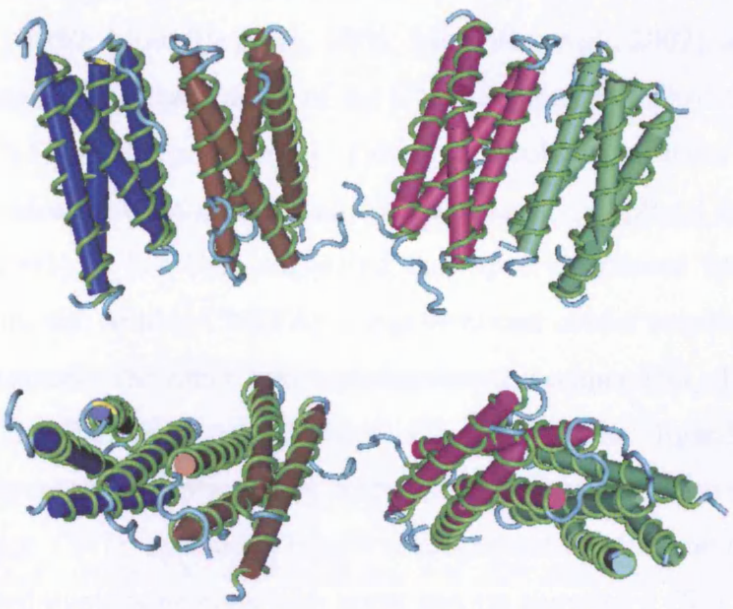
Neurotrophic factors have been assessed for their effects on rubrospinal tract regeneration as well as red nucleus survival and atrophy. Research has been focused primarily on the effects of BDNF, CNTF and NT3 with a few studies examining the role of NT4/5, GDNF and FGF2. Most of the studies involving the use of neurotrophic factors to induce regeneration of the lesioned rubrospinal tracts are focused on adult rather than neonate animals.

5.1.4.1 CNTF

As discussed in section 5.1.2.2 (page 257), a rubrospinal tract lesion is accompanied by changes in expression of various molecules detected in red nucleus somata. Like other CNS neurons that are involved in motor control, intact red nucleus neurons are positive for the presence of the CNTF receptor (CNTFRα) (Davis *et al.*, 1993; Ip *et al.*, 1993; Curtis *et al.*, 1994; MacLennan *et al.*, 1996) and could therefore be responsive to exogenous CNTF. However, in line with other neurotrophic factors, CNTF has a very short serum half-life of about ≈3 minutes, does not cross the BBB and can cause significant cytokine-like effects if administered systemically (Dittrich *et al.*, 1994). These limitations suggest that delivery by means of viral vectors should be particularly effective, allowing the *de novo* synthesis of CNTF by red nucleus neurons. Furthermore, CNTF is retrogradely transported to the neuronal somata and the rate of this transport has been shown to increase following an axotomising sciatic nerve lesion (Curtis *et al.*, 1993). The CNTF gene employed in the experiments described in this Chapter, encodes a secretable form of this factor as it has a secretory signal (discussed later). It is therefore possible that being able to synthesise the factor by the axotomised red nucleus neurons themselves, would add to its beneficial potential by also acting in adjacent neuronal cell populations.

CNTF has been shown to support the survival of motor neurons both *in vitro* (Ip *et al.*, 1991; Sendtner *et al.*, 1992; Sendtner *et al.*, 2000) and *in vivo* (Friedman *et al.*, 1992). Following cord hemisection at T2, CNTF and CNTFRα mRNA levels increase in motor neurons within 1 hour post injury and then rapidly decline to subnormal levels by day 10 (Mata *et al.*, 1993; Oyesiku *et al.*, 1997). The initial burst of CNTF mRNA expression is seen both rostral and caudal to the lesion whereas CNTFRα mRNA upregulation is confined to motor neurons caudal to the lesion site (Oyesiku *et al.*, 1997). The parallel pattern of CNTF/CNTFRα upregulation is in line with the role of CNTF as a survival and regeneration-promoting factor for local injured motor neurons and that may be released from axotomised neurons or reactive astrocytes surrounding the lesion site (Sendtner *et al.*, 1992; Oyesiku and Wigston, 1996; Oyesiku *et al.*, 1997). CNTF may also have a stimulating effect on mature astrocytes leading to gliosis in the adult brain

A. Predicted structure of human CNTF



B. Predicted binding domain of human CNTF - Rα

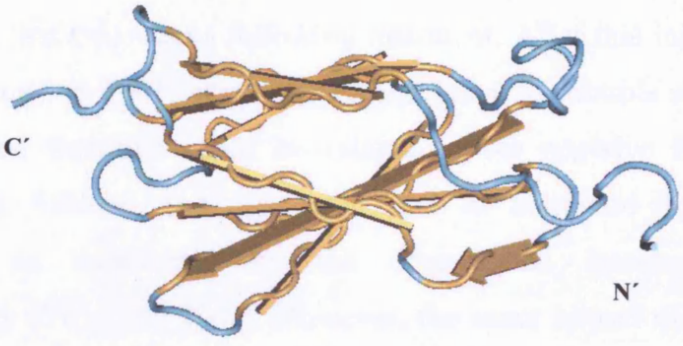


Figure 5.1.4-a: Predicted structure of CNTF & its CNTF-Rα binding domain.

Three dimensional representations of the predicted structures of **A:** the four domains of human CNTF (GI: 25952136, MMDB5903) (McDonald *et al.*, 1995) and **B:** the C′-terminal domain of the human CNTF receptor (CNTF-Rα) (GI:180710, MMDB29135) which is the binding site for human CNTF (Man *et al.*, 2003; He *et al.*, 2005).

(Clatterbuck *et al.*, 1996; Levison *et al.*, 1996; Lisovoski *et al.*, 1997). Mature astrocytes are able to respond to CNTF despite their lack of CNTFRα expression. This is potentially due to the illegitimate binding of CNTF to the leukaemia inhibitory factor receptor (LIFR), present on astrocytes (Sendtner *et al.*, 1990; Friedman *et al.*, 1992; Monville *et al.*, 2001; Monville *et al.*, 2002). In addition, the local increase in the soluble form of the CNTFRα may still play a role in the regulation of CNTF activity as it might function as a soluble mediator for CNTF, since it can be released from muscle cells in response to peripheral nerve injury (Davis *et al.*, 1993). It has been suggested that upon its release from the cell plasma membrane the soluble CNTFRα component can confer responsiveness to cells that express only the other two transmembrane components of the CNTF receptor and are therefore unresponsive to CNTF. The ligand to these "incomplete" receptors is formed only when CNTF associates with the soluble CNTFRα receptor. CNTF appears to have a neuroprotective effect on the survival of axotomised red nucleus neurons with some reports showing a CNTF mediated functional recovery. For example, intrathecal administration of recombinant CNTF over a 10-day period following a contusion lesion at the T10 level, increases the number of red nucleus neurons that remain capable of retrograde transport (Ye *et al.*, 2004). In terms of recovery of function, Ye and colleagues (2004), reported that these animals displayed some improvements in motor skills, but only for the first two weeks following treatment. After this initial period no further improvement in CNTF treated animals relative to controls was noted. The authors suggested that this could be related to the negative impact of the astrogliosis that follows such an insult and is amplified by the CNTF administration, as manifested by the dose-related increase in GFAP immunoreactivity (Ye *et al.*, 2004). However, the exact impact of this response remains somewhat unclear. The beneficial effects of CNTF on axotomised lesioned red nucleus neurons as seen in the contusion experiments of Ye *et al.* (2004), were replicated when CNTF was administered to animals with a chronic axotomy. Administration of recombinant CNTF, 8 weeks after an upper cervical rubrospinal tract lesion, enhanced regeneration of injured rubrospinal neurons into a peripheral nerve graft (Houle and Ye, 1997). In this model however, the response was complicated by the fact that placing a CNTF-soaked gel foam pad

within the lesion site caused a second axotomising lesion that also plays a role in the response of red nucleus axons to this factor. Thus when a secondary rubrospinal tract lesion is followed by the administration of CNTF, there is an increased survival of red nucleus neurons (Houle and Ye, 1999), accompanied by a greater increase in the extent of c-Jun upregulation than that is seen with re-injury alone (Houle *et al.*, 1998).

5.1.4.2 BDNF

The decision to construct a BDNF-expressing HSV1 based vector targeting the injured rubrospinal tract was based on a series of studies that are briefly reviewed in the paragraphs that follow. These studies document the ability of this neurotrophic factor to rescue red nucleus neurons from axotomy-induced atrophy (Plunet *et al.*, 2002; Liu *et al.*, 2002; Kwon *et al.*, 2002; Storer *et al.*, 2003; Lynskey *et al.*, 2006).

The increased rubrospinal tract survival achieved with the administration of BDNF (Kobayashi *et al.*, 1997; Kwon *et al.*, 2002) reflects changes in the expression of regeneration and survival-associated genes. In contrast to the response seen in injured motor neurons, the expression of TrkB in axotomised rubrospinal neurons declines over the subsequent weeks following a lesion (Novikova *et al.*, 2000; Kwon *et al.*, 2004). On closer analysis, however, it appears that TrkB expression within red nucleus neuronal cell bodies never completely ceases. Low levels of full length TrkB immunoreactivity were present in the cell bodies of axotomised red nucleus neurons even at 12 months following axotomy (Kwon *et al.*, 2002). This was also the case in a contusion model of rubrospinal tract injury. In this paradigm, full length TrkB-IR was still present in the red nucleus somata at 42 days post injury. At the site of the lesion and in degenerating white matter, the truncated form of the receptor was upregulated (King *et al.*, 2000; Liebl *et al.*, 2001). This increased expression of the truncated form of TrkB has been mapped to the oligodendrocytes and astrocytes that surround the lesion site (Frisen *et al.*, 1992; Frisen *et al.*, 1993).

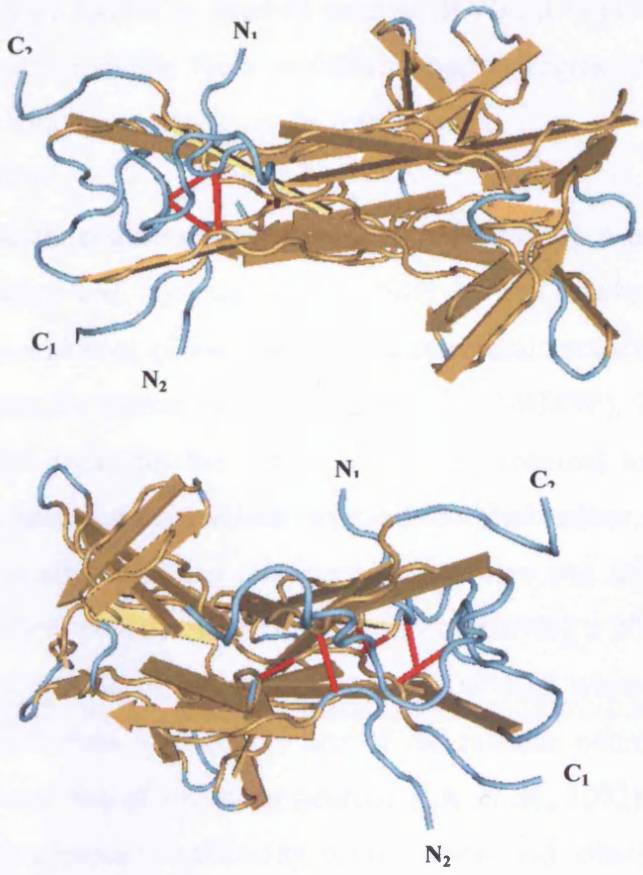
The continuous infusion of BDNF (≈10 μg/day) into the subarachnoid space during postoperative weeks 5-8, appears to prevent the axotomy-induced decline of full length TrkB in red nucleus neuronal somata (Novikova *et al.*, 2000). This is in line with reports that the administration of BDNF in close proximity to the red nucleus neuronal somata is able to reverse the axotomy-induced atrophy (Kwon *et al.*, 2002). In contrast, delivering BDNF close to the lesion site 2 months following a cervical level rubrospinal tract axotomy, failed to prevent the subsequent neuronal atrophy or promote any axonal regeneration into a peripheral nerve graft (Kwon *et al.*, 2004). Interestingly, the application of BDNF at the lesion site was capable of preventing the increase in the expression of the truncated form of the TrkB receptor found in oligodendrocytes and astrocytes surrounding the lesion site of a thoracic level spinal cord hemisection (King *et al.*, 2000).

The studies described above appear to suggest that targeting of BDNF delivery to neuronal cell bodies may be more efficient at promoting red nucleus neuron survival and axonal regeneration than delivery to axons. Further evidence supporting this suggestion comes from the fact that exposure of red nucleus somata to high concentrations of BDNF leads to an increase of GAP43 and Tα1-Tubulin expression at the site of axonal injury. This upregulation in regeneration-associated gene expression may correlate with an increased ability of rubrospinal axons to regenerate into a peripheral nerve graft, that is induced by the BDNF treatment (Kobayashi *et al.*, 1997; Kwon *et al.*, 2002). It is interesting to note that neither GAP43 nor Tα1-Tubulin mRNA levels are upregulated when BDNF is administered at the site of a cervical level rubrospinal tract lesion (Kwon *et al.*, 2004). The lack of up-regulation of these two molecules does not imply that red nucleus neurons are totally refractory to BDNF when it is administered at the lesion site. Infusion of BDNF near a unilateral C4 rubrospinal axotomy site up-regulates the expression of another molecule: Gal-1 (McGraw *et al.*, 2004b) which as discussed in section 5.1.2.2, declines following an rubrospinal tract axotomising lesion. It has been suggested that the oxidized form of Gal-1 (Gal-1/OX) may positively correlate with the ability of motor as well as sensory neurons to initiate a regenerative response (Horie and Kadoya, 2004; McGraw *et*

al., 2004a; McGraw *et al.*, 2004b). Even though the mechanism of Gal-1/OX action is still unclear it is thought that at least in the PNS it stimulates macrophages to secrete a factor that is involved in axonal growth and/or Schwann cell migration leading to enhanced regeneration (Fukaya *et al.*, 2003; Horie *et al.*, 2004).

Overall, the administration of BDNF, be it near the somata or at the lesion site, has the potential to influence red nucleus neuronal survival and axonal regeneration, although greater effects are to be expected if BDNF is made available in the red nucleus. Several reports support the potential of BDNF within this context. These involve experiments employing BDNF infusions, OECs, genetically engineered fibroblasts or direct transduction with viral vectors. Normal OECs have been shown to be unable to promote significant rubrospinal axonal regeneration in the spinal cord (Ruitenberg *et al.*, 2003; Ramer *et al.*, 2004). However, when primary OECs were transduced by means of an Adenoviral Vector (AdV) to express rBDNF and were subsequently implanted at the site of a C4 rubrospinal tract lesion more rubrospinal tract sprouting, together with regeneration into the lesion site was observed (Ruitenberg *et al.*, 2003). It should be emphasised that even though this pattern of regeneration was reported at the four month time point, the AdVrBDNF vector enabled the rBDNF expression from transduced OECs for a maximum of 30 days. Ruitenberg *et al.* (2003) noted that relatively few rubrospinal tract axons entered the lesion site. The majority of sprouting axons remaining at the lesion site/graft interface and this was attributed to the presence of inhibitory factors within the lesion site (Fawcett and Asher, 1999; Ruitenberg *et al.*, 2003; Sandvig *et al.*, 2004). In another study by Xiao et al. (2005), olfactory neuroepithelium (ONe) derived - neurosphere forming cells (NSFCs) shown to constitutively secrete BDNF, were transplanted at the site of a C3-4 unilateral rubrospinal tract lesion. Two weeks following transplantation, red nucleus neuronal somata appeared to have evaded atrophy. There was only a 7% reduction of mean somal area in treated animals as opposed to a 27% reduction in control animals. In terms of axonal regeneration, rubrospinal tract axons were reportedly able to regenerate into and through the ONe-NSFC transplant and extend into the spinal cord for 3-4 segments caudal to

A. Predicted BDNF structure



B. Predicted structure of the TrkB receptor, BDNF binding site

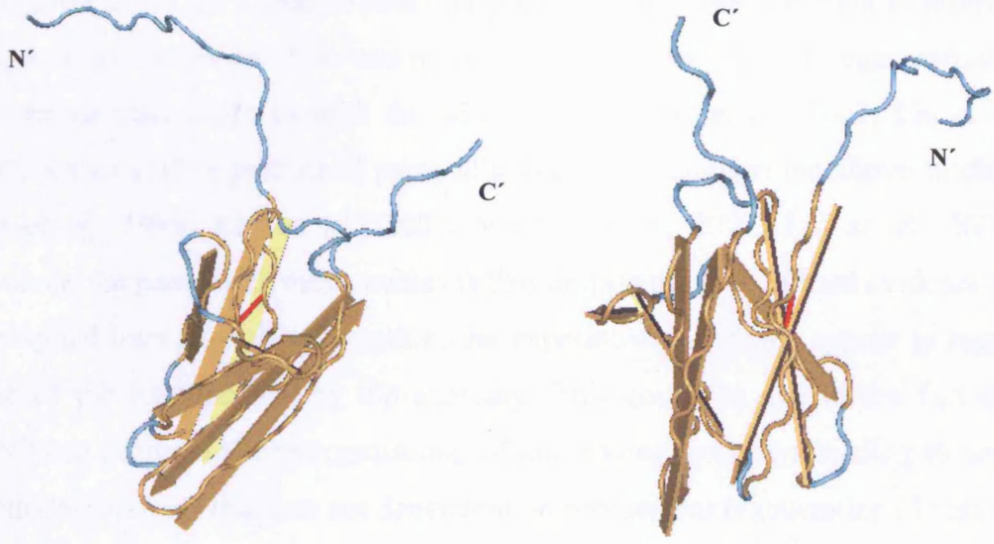


Figure 5.1.4-b: Predicted structures of BDNF & its binding domain on the TrkB receptor.

Three dimensional representations of the predicted structures of **A**: the A chain of human BDNF (GI:584184, MMDB9670) and **B**: the 5th domain (d5) of the human TrkB receptor (GI:5822440, MMDB10999) (Ultsch *et al.*, 1999) which is the binding site for human BDNF (Urfer *et al.*, 1995). Structures were obtained using the Cn3D4.1 software, available at the NCBI website: http://www.ncbi.nlm.nih.gov (Disulfide bridges are shown in red).

the lesion (Xiao *et al.*, 2005). It should be pointed out though that despite the fact that ONe-NSFCs were shown to innately secrete BDNF, it is possible that they also secrete factors that enable them to further support regeneration and allow axons to evade the inhibitory signals of the lesion site.

Fibroblasts, genetically engineered to express BDNF have been used with some success in promoting red nucleus neuron survival and axonal regeneration following axotomy. Liu *et al.* (2002) employed a retroviral vector to transduce rat fibroblasts to express the human version of BDNF (Fb/hBDNF). Transplantation of these Fb/hBDNF cells into the site of a C3-4 rubrospinal tract lesion was shown to limit the extent of red nucleus neuronal loss and reduce somal atrophy. Specifically, two months following this intervention there was reportedly a 15% red nucleus neuron loss with the surviving neurons displaying a 20% reduction of somal size. The magnitude of this effect becomes evident when one considers that in control animal there was a 45% loss of red nucleus neurons and a 40% reduction in the somal size of surviving neurons (Liu *et al.*, 2002). However, the effectiveness of this approach in reducing neuronal loss and somal atrophy is not mirrored in terms of rubrospinal tract axonal regeneration. Even though transgene expression lasted for 2 months post transplantation and was sufficient to promote a regenerative response, there was no persuasive evidence for the regeneration of rubrospinal tract axons through the lesion site (Murray *et al.*, 2002; Liu *et al.*, 2002). Other studies performed using this technology support the above findings (Liu *et al.*, 1999; Kim *et al.*, 2001; Murray *et al.*, 2002; Liu *et al.*, 2002). However, the paradox of these studies is that despite the lack of hard evidence for rubrospinal tract axonal regeneration, the experimental animals appear to regain some of the function lost by the axotomy. This could be due to the fact that BDNF can promote the re-organisation of descending pathways leading to some functional recovery that was not dependent on rubrospinal regeneration (Tobias *et al.*, 2005).

One problem with the therapeutic use of BDNF is that it has the shortest plasma half-life of all other neurotrophic factors at ≈0.92 minutes and does not cross the intact BBB (Poduslo and Curran, 1996). These characteristics may hinder its

application *in vivo*. The application of HSV1 viral vector technology however can help circumvent this limitation and exploit the regeneration promoting abilities of this factor. The potential of the HSV1.pR19CMV vector backbone employed in this study to facilitate the *de novo* synthesis of BDNF from red nucleus neuron somata would complement the suggested mode of action of this factor in these neurons. The same principle has shown promise when applied by means of a BDNF expressing adeno-associated virus (AAV) vector. Ruitenberg *et al.* (2004) showed that the direct administration of AAV-BDNF at the level of the red nucleus at the time of the lesion cannot prevent the onset of neuronal atrophy but it has the potential to reverse the atrophy that follows a cervical rubrospinal tract axotomising injury. Specifically, at the two month time point following AAV-BDNF administration, the mean red nucleus neuron somal area was ≈395 μm^2 in AAV-BDNF treated animals as opposed to ≈251 μm^2 in AAV-GFP, control animals (Ruitenberg *et al.*, 2004). The highly disabled HSV1 vector used in this study could in theory achieve a similar outcome, especially since it preferentially targets neurons where it can establish a long term and stable expression of BDNF.

Other tissues that secrete BDNF have differing effects on injured rubrospinal neurons. For example, transplantation of foetal spinal cord segments into the site of a C3-4 rubrospinal tract axotomising lesion, can lead to a ≈50% reduction in cell loss but does not lead to any meaningful rubrospinal axonal regeneration into or through the lesion site (Mori *et al.*, 1997). On the other hand, rubrospinal axons can regenerate into a peripheral nerve graft placed within the cervical spinal cord (Richardson *et al.*, 1984).

5.1.4.3 Neurotrophin 3

Exogenous administration of NT3 has been shown promote the survival of axotomised red nucleus neurons in newborn rats but only in the short term (Diener and Bregman, 1994). In the adult animal, NT3 encourages the axonal elongation of red nucleus neurons into a peripheral nerve graft placed rostral to the lesion, four weeks after an upper cervical axotomy. (Ye and Houle, 1997).

When fibroblasts engineered to stably express NT3 were transplanted into a cervical over-hemisection, which included the rubrospinal tract, some recovery of function occurred over the following three months (Grill *et al.*, 1997). However, this may not have been purely due to the effects of NT3. Evidence supporting this comes from studies employing a different substrate to regeneration. For example, combining the use of embryonic transplants and co-administration of NT3 one month following a C5/C6 rubrospinal tract axotomy, appeared to encourage axons to sprout at the margin of the lesion site but with minimal growth into the transplant (Lynskey *et al.*, 2006). Despite the somewhat expected, marginal nature of the axonal response, it was nevertheless accompanied by improvements in motor function probably due to the plasticity of local, spared fibres (Grill *et al.*, 1997; Shumsky *et al.*, 2003; Tobias *et al.*, 2003; Lynskey *et al.*, 2006). After all, spared CST axons in the vicinity of an rubrospinal tract axotomising lesion have been shown to be capable of some sprouting in the presence of exogenously derived NT3, albeit only in the superficial dorsal horn (Jeffery and Fitzgerald, 2001) and this phenomenon may underlie the improvements in motor function observed in these experiments. This is supported by the fact that in the case of a complete unilateral C3/C4 hemisection that eliminates the ipsilateral motor pathways, no improvements in motor function were observed in BDNF/ NT3 treated animals (Shumsky *et al.*, 2003). Since this type of lesion ablates the descending motor and pre-motor neuronal projections that regulate the function of forelimb muscle groups, it follows that any improvement noted in models that spare these tract could be due to spared ipsilateral fibres (Shumsky *et al.*, 2003).

The above studies appear to suggest that NT3 may not be as efficient as other factors in promoting the regeneration of axotomised red nucleus neurons. This is supported by the fact that NT3 treatment, administered either at the lesion site or at the level of the red nucleus cell bodies, is not associated with an increased expression of GAP43 and α1 Tubulin. At the same time there is only a marginal increase in full-length TrkC mRNA levels at 7 days following a cervical rubrospinal tract lesion (Tetzlaff *et al.*, 1991; Kobayashi *et al.*, 1997; King *et al.*, 1999). In addition, administration of NT3 alone does not protect against atrophy to the extent observed after BDNF infusion (Kobayashi *et al.*, 1997; Houle and

Ye, 1999). This does not mean that red nucleus neurons are totally unresponsive to NT3. Prolonged administration of NT3 appears to have a restorative effect on TrkB levels which decline following a rubrospinal tract axotomy (Novikova *et al.*, 2000). Treatment with NT3 following a C3 level lesion, augments the expression of mRNA transcripts of the β1 subunit of the integrin receptor in red nucleus neurons, a putatively pro-regenerative response (Plantman *et al.*, 2005). No such upregulation occurs as a result of BDNF administration. NT3 alone, and to greater extent NT3 administration combined with a foetal spinal cord transplant to a thoracic level rubrospinal tract lesion site, leads to an increase in the expression of c-Jun (Broude *et al.*, 1999).

The combination of BDNF and NT3 infusions at 5-8 weeks following axotomy, can lead to reductions in red nucleus somal atrophy following a cervical level lesion, pointing to a potentially synergistic relationship between those two factors (Novikova *et al.*, 2000). When the same combination was infused acutely and following a C3 lesion it failed to have an impact on neuronal somata. The authors of this study deduced that the extent of the effect of these neurotrophins depends on the timing of their administration with respect to the initial injury. This however is in contrast with reports based on the use of modified BDNF and NT3 expressing fibroblasts. For example, Tobias *et al.* (2003) report that it is the acute rather than delayed (by 6 weeks) transplantation of BDNF and NT3 fibroblasts that produce a maximal effect in terms of preventing red nucleus cell body atrophy. There is therefore a disagreement on the correct timing of this intervention with one group favouring the acute (Tobias *et al.*, 2003) and the other the delayed (Novikova *et al.*, 2000) intervention with a combination of BDNF and NT3. This discrepancy could be explained by taking into consideration the significant differences between research groups with respect to the methods employed in order to identify, count and measure the somal size of axotomised red nucleus neurons (Tobias *et al.*, 2003).

5.1.4.4 GDNF

In addition to the studies described previously, there have been a few studies on the potential therapeutic effects of other factors after lesions of the rubrospinal tract. Following a lesion of the rubrospinal tract at C3 and a subsequent 60-minute exposure to recombinant GDNF within the lesion site, *in situ* hybridisation analysis revealed upregulation of βIII Tubulin mRNA levels within the first three days following exposure (Storer *et al.*, 2003). In addition, GAP43 mRNA also increased, accompanied by an increase in red nucleus neuronal cell body size. It is worth noting that GDNF was applied to a second aspiration lesion, which was in turn carried out four weeks after the initial lesion. It is therefore plausible that the increase in mRNA levels noted was due to the effects of the second lesion inflicted rather the result of the GDNF administration *per se*.

In a previous study the same research group report that βIII Tubulin mRNA levels are increased within 6 hours after a secondary axotomy and remain stable for the next 5 days without any further intervention (Storer and Houle, 2003). The same authors report that GAP43 mRNA levels also increase within six hours after the secondary lesion and reach a steady state within the subsequent 7 days. The results from these studies however must be interpreted with caution because, as the authors acknowledge, there is a lack of statistical significance in their findings (Storer *et al.*, 2003).

In another experiment, GDNF was administered acutely after an axotomising lesion by means of a gel foam application (Dolbeare and Houle, 2003). These authors report that at one week following the initiation of GDNF treatment, there is a marked reduction in the extent of dieback of injured axons. Furthermore, after a month-long treatment, rubrospinal tract axons were present ≈240 μm from the rostral margin of the lesion site compared to ≈480μm in PBS treated animals (Dolbeare and Houle, 2003).

5.1.4.5 bFGF

Several research groups have attempted to map the presence of bFGF and its receptor in distinct neuronal populations including the red nucleus. Uninjured, adult red nucleus neurons are immunopositive for the presence of bFGF (Grothe *et al.*, 1991; Matsuyama *et al.*, 1992; Grothe and Janet, 1995; Fuxe *et al.*, 1996). No data are as yet available as to the expression of bFGF and its receptor after lesion of the rubrospinal tract and very few attempts have been made to examine the potential of this growth factor to promote the regeneration of axotomised red nucleus neurons. Local application of bFGF, accompanied by removal of the glial scar, at either 4 or 8 weeks following a lesion at the C3 level, fails to promote an increase in the levels of c-Jun in red nucleus neurons (Houle *et al.*, 1998). In another study using the same lesioning model, the application of bFGF at four weeks following the initial lesion had no effect on the survival of axotomised red nucleus neurons (Houle and Ye, 1999).

5.1.5 Rationale

This study describes experiments employed to test the hypothesis that if vectors HSV1.pR19CMVrBDNF and HSV1.pR19CMVmCNTF are capable of transducing injured RST neurons to *de novo* synthesise biologically active rBDNF and mCNTF, then these constructs may lead to a more favoured regenerative response in injured RST neurons. This relationship could be measured as reduced somal atrophy in red nucleus neurons when using the HSV1.pR19CMVrBDNF vector and increased axonal regeneration when targeting the lesioned rubrospinal tract model with both HSV1.pR19CMVrBDNF and HSV1.pR19CMVmCNTF.

5.2 Methods

5.2.1 Vectors utilised in this Chapter

The following vectors were used in this study, either to induce regeneration or for the delivery of marker genes to the chronically injured rubrospinal tract.

Virus	Abbreviation
1764/ICP27$^-$/ICP4$^-$/RL1$^-$/pR19CMVGFP	HSV1.pR19CMVGFP
1764/ICP27$^-$/ICP4$^-$/RL1$^-$/pR19CMVrat BDNF	HSV1.pR19CMVrBDNF
1764/ICP27$^-$/ICP4$^-$/RL1$^-$/pR19CMVmouse CNTF-NGF chimera5	HSV1.pR19CMVmCNTF

Table 5.2-a: Vectors used in rubrospinal tract regenerative studies.

The construction and characterisation of the vector backbone HSV1.pR19CMV has been previously published (Lilley *et al.*, 2001b) and characterised *in vivo* in Chapter 3.

5.2.2 Construction of rBDNF or rCNTF expressing vectors

As discussed in Chapter 3, the vector backbone that allows efficient transgene delivery to the chronically injured spinal cord was 1764/ICP27$^-$/ICP4$^-$/pR19CMV (HSV1.pR19CMV). As is the case for other neurotrophin expressing vectors, such as those described in Chapter 4, in order to create vectors capable of delivering biologically active rBDNF or mCNTF proteins, the corresponding cDNA had to be first sub-cloned into the pR19CMV expression cassette, substituting the GFP marker gene. Insertion of the new expression cassette into the host viral genome was subsequently achieved via homologous recombination. Production of functional protein and confirmation of transgene product bioactivity was then confirmed *in vitro* and *in vivo*.

5.2.2.1 Construction of plasmid pGEM5/pR19CMVrBDNF

cDNA encoding for rBDNF (Gene Bank accession number M61175) was obtained from Dr B. Haupt (Dept. of Immunology & Molecular Pathology, UCL, London). The cDNA was originally cloned as an *EcoR*I, PCR generated fragment (Maisonpierre *et al.*, 1991) into pSP72/rBDNF plasmid. The reporter gene GFP of the plasmid backbone pGEM5/pR19CMVGFP, was removed by digesting first with *Hind*III followed by blunt ending with T4 DNA polymerase and subsequent restriction with *Xho*I. A single rBDNF corresponding fragment (≈1137bp) was removed from pSP72/rBDNF by digesting with *EcoR*V/*Xho*I. The fragment was

then sub-cloned into the pR19CMV shuttle plasmid, flanked by LAT sequences which allow for the subsequent homologous recombination into the LAT region (Lilley *et al.*, 2001b). pGEM5/pR19CMVrBDNF clones were screened by a single *EcoR*I digestion. Clones containing the rBDNF gene produced a single ≈1127bp band (Maisonpierre *et al.*, 1991) and were selected on the basis of having this restriction profile.

5.2.2.2 Construction of plasmid pGEM5/pR19CMVmCNTF

The mCNTF cDNA (Gene Bank accession number NM_170786) utilised for the production of the recombinant vector employed in this study was a kind donation from Prof M. Sendtner (Dept. of Neurochemistry, Max-Planck-Institute for Psychiatry, Martinsried, Germany), in the form of plasmid pCNTF-chim5 encoding a secretable form of mCNTF obtained by the fusion of the murine NGF-β subunit secretory signal with the mCNTF cDNA (Sendtner *et al.*, 1992). The mCNTF chimeric protein is therefore capable of being exported from transduced cells. The cDNA fragment (2150bp) was excised from the pCNTF-chim5 plasmid via an *Xba*I/T4/*Hind*III digest and sub-cloned into pGEM5/pR19CMVGFP. The GFP reporter gene was previously removed from the same plasmid backbone via a *Xho*I/T4/*Hind*III restriction. pGEM5/pR19CMVmCNTF recombinant clones were screened for the presence of mCNTF via a *Hind*III/*Xmn*I digestion which yielded a single ≈1200bp band. Out of all the positive clones a single colony was selected and further confirmed by sequencing.

5.2.2.3 Production of HSV1.pR19CMVrBDNF and mCNTF vectors

Purified HSV1.pR19CMVGFP (1764/ICP27$^-$/ICP4$^-$/pR19CMVGFP) viral DNA was co-transfected with pGEM5/pR19CMVrBDNF or pGEM5/pR19CMVmCNTF plasmid DNA. Each of the constructs was recombined into the endogenous LAT of the HSV1.pR19CMVGFP backbone between the two *BstXI* sites (*nt*120,220 and *nt*120,408) (Lilley *et al.*, 2001b). Recombinant plaques were detected by their white phenotype (no GFP expression), which is indicative of the correct insertion of the pR19CMVrBDNF

or mCNTF expression cassettes in both LAT regions of the HSV genome. Plaque purification was carried out until all plaques had the recombinant, white phenotype. The two new vectors were termed HSV1.pR19CMVrBDNF (1764/ICP27$^-$/ICP4$^-$/pR19CMVrBDNF) and HSV1.pR19CMVmCNTF (1764/ICP27$^-$/ICP4$^-$/pR19CMVmCNTF). Southern blotting confirmed the correct insertion of the rBDNF gene on recombinant plaques, each originating from separate recombination events. Recombinant vectors were propagated by standard cell culture techniques on 27/12/M:4 cells and viral stocks were produced as outlined in Chapter 2.

5.2.3 Characterisation of HSV1.pR19CMVrBDNF & mCNTF vectors

Correct transgene insertion and the ability of these vectors to secrete biologically active protein was evaluated using a non-complementing cell line.

5.2.3.1 Southern blotting on HSV1pR19CMVrBDNF plaques

Each purified recombinant HSV1.pR19CMVrBDNF viral plaque was used to infect 1x10^6 BHK cells. Viral DNA from each plaque was extracted as outlined in Chapter 2, digested with *EcoR*I and run on a 2% (w/v) agarose gel. HSV1.pR19CMVGFP viral DNA digested by *Hind*III/*Xho*I (releasing a 600bp fragment corresponding to GFP) was used as a negative control while an *EcoR*I digested plasmid pSP72/rBDNF was used as a positive control. The same plasmid digest was used to produce the rBDNF probe, which was subsequently purified and radiolabelled with αP^{34}-dCTP. A detailed account of this procedure is given in Chapter 2.

5.2.3.2 Southern blotting on HSV1.pR19CMVmCNTF plaques

The purification procedure was carried out as outlined above. In the case of HSV1.pR19CMVmCNTF plaques, the extracted viral DNA was digested with *Hind*III/*Xmn*I (releasing a ≈951bp fragment corresponding to the 5' end of mCNTF which includes the NGF secretory sequence). HSV1.pR19CMVGFP viral DNA, digested as described above, was used as a negative control, while a *Hind*III/*Xmn*I digested plasmid pmCNTFchim5 was used as a positive control.

5.2.3.3 Concentration of rBDNF/mCNTF from transduced BHKs

$1x10^6$ BHK cells in a 36 mm well were infected at an MOI of 1 with either HSV1.pR19CMVGFP or neurotrophic factor encoding vector: HSV1.pR19CMVrBDNF or HSV1.pR19CMVmCNTF. Two days later, the infected monolayer was washed twice with 0.1M PB and 500 µl of DMEM was placed in each well. The now infected cells were returned to a 37°C/5%CO_2 incubator. At the desired time point, the DMEM was collected and centrifuged at 2,000 rpm for 5 minutes to remove any cells present. The supernatant was then transferred onto a 500 µl Microcon column with a 10kDa molecular weight cut-off (G-10000, Green). The column was then spun at 11,500g for 25 minutes at 4°C. The total volume of the concentrated sample was 20-25 µl, which represents the amount of recombinant protein, rBDNF or mCNTF, secreted from $1x10^6$ virally transduced, non-complementing cells. These were used for further testing via Western blotting or neurite outgrowth assay.

5.2.3.4 Western blotting for vector rBDNF/ or mCNTF detection

The Western blotting analysis carried out in this Chapter involved screening for the presence of rBDNF or mCNTF in conditioned media as well as, in some instances, their presence in cell lysates. Both techniques employed have been extensively described in previous chapters (Chapters 2 and 4), while protein isolation from conditioned media is outlined in the previous section. rBDNF expression was analysed at 2, 4, 6 and 8-hour time points in conditioned media and at a single time point of 8 hours in the case of whole cell lysates. Secreted mCNTF, expression was assessed in conditioned media, at a single time point of 6 hours. Cell lysates or concentrated samples from conditioned media were mixed with SDS-PAGE sample buffer and processed as described in Chapter 2. In the case of the mCNTF electrophoresis, the sample buffer used did not contain any β-mercaptoethanol. Instead it contained DTT at a final concentration of 2mM in order to partially preserve disulfide bonds and dimer integrity. The primary antibody used for the detection of rBDNF was rabbit polyclonal anti-human BDNF (working dilution 1:200, N-20, Santa Cruz Biotechnology Inc., SC-546), which cross-reacts with the rat species of the protein and significantly recognises

the unprocessed 32 kDa pro-BDNF, the 28 kDa intermediate and the 14.5 kDa mature BDNF proteins. For detecting mCNTF, the primary antibody used was goat polyclonal anti-rat CNTF (working dilution 1:200, R-20, Santa Cruz Biotechnology, Inc., SC-1912) which also cross-reacts with CNTF of murine origin. Equal protein loading was confirmed by visually evaluating band intensity on a duplicate, coomassie G-250 stained gel.

5.2.3.5 rBDNF neurite outgrowth assay on E14 rat DRG explants

This assay was described in detail in section 4.2.2.7 of Chapter 4. Briefly, rat E14 lumbar DRGs were isolated, embedded in collagen and maintained in a chemically defined medium. The supernatant from $1x10^6$ BHK cells previously infected at an MOI of 1 with HSV1.pR19CMVrBDNF (n=3) or HSV1.pR19CMVGFP (n=3) (negative control) or mock-infected (n=2) was collected, clarified to remove cellular debris and concentrated as described in section 5.2.3.3. Concentrated, conditioned medium was added to each collagen-embedded DRG explant and replenished every day. Neurite extension was compared 3 days following the initiation of treatment by immunofluorescence staining of neurites using mouse TUJ1 anti-neuron specific type III β tubulin antibody (1:5000, Cambridge Bioscience, UK). Recombinant human BDNF (R&D Systems, 248-BD) was used as a positive control for the experiment at a concentration of 5 ng/ml.

5.2.4 Cell proliferation assay

This assay was employed in order to confirm the biological activity of vector encoded mCNTF and was based on the use of the cell line TF-1.CN5a.1 (ATCC®, Cat. No: CRL-2512). These cells have been engineered to express the alpha subunit of the human CNTF-Rα receptor and proliferate in the presence of minute quantities of biologically active CNTF (in the picogram/ml range). $1x10^6$ BHK cells in a 36 mm well were infected at an MOI of 1 with either HSV1.pR19CMVGFP or HSV1.pR19CMVmCNTF. Two days later the infected monolayer was washed twice with 0.1M PB, aliquots of 500 µl of DMEM were placed in each well and the infected monolayer was returned to a 37°C/5%CO_2

incubator overnight. The following day the supernatant was removed and spun at 12,000 rpm at 4°C for 30 min to pellet cell debris or free viral particles. In order to confirm the removal of virus, 10µl of this supernatant was titrated on the complementing cell line 27/12/M:4 and the cells were monitored for the appearance of viral plaques. Purified conditioned supernatants were kept at 4°C and used immediately after purification. 5, 25 and 50 µl aliquots from each conditioned media was analysed in triplicate for its ability to induce the proliferation of $3x10^4$ TF-1.CN5a.1 cells. Conditioned media from HSV1.pR19CMVGFP infected cells served as a negative control while 2ng/ml of recombinant human CNTF (rhCNTF, R&D systems, Cat.No: 257-NT) was used as a positive control. Cell numbers were monitored at three time points: prior to the addition of conditioned media or rhCNTF (t/p0) and at 3 and 6 days following sample addition (t/p3 and t/p6 respectively). Cell numbers were assessed using a haemocytometer and Microsoft® Excel was utilised for analysis.

5.2.5 HSV1.pR19CMV mediated *in vivo* delivery of rBDNF or mCNTF

Two sets of experiments were carried out during the course of these studies. The first set was designed to examine the effect of vector-mediated rBDNF and mCNTF delivery on lesioned rubrospinal tract axons (section 5.3.3, page 300). The second set aimed to assess the ability of vector mediated rBDNF delivery on red nucleus axotomy induced somal atrophy (5.3.4, page 306). The above experiments are represented schematically in Figure 5.2.5-a and Figure 5.2.5-b respectively.

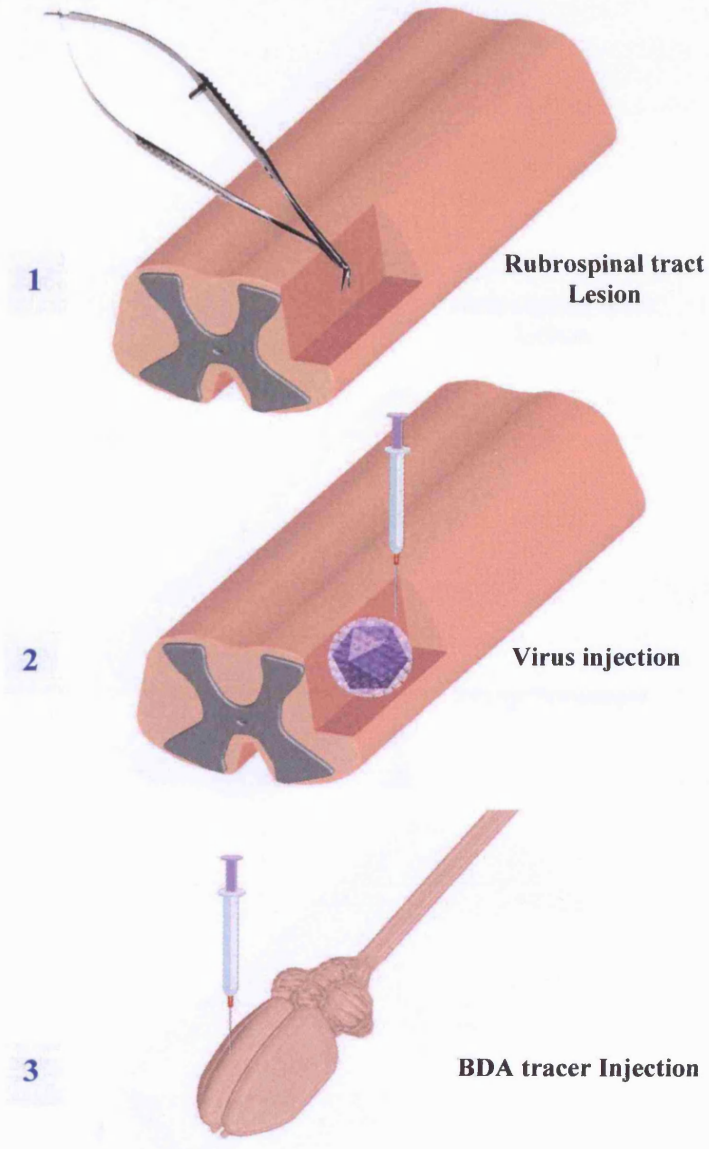
5.2.5.1 Rubrospinal tract lesions

Lesions were performed on deeply anaesthetised adult female Lewis rats (≈200g). A horizontal incision was made in the skin at the midline over the thoracic spine. A second incision was made in the superficial muscle layers and the underlying muscles were separated by blunt dissection. The mid-thoracic vertebrae (≈T4 to T6) were exposed and a bilateral laminectomy, performed using malleus scissors. The dura mater was carefully cut with micro-scissors and at this point, animals were injected subcutaneously with 2.2 mg/Kg of Finadyne (Schering-Plough).

With the aid of iridectomy scissors, the left lateral funiculus was transected. This severed the descending rubrospinal tract, sparing the ascending dorsal columns and the descending dorsal corticospinal tract. The dura matter and overlying musculature was repaired using silk sutures and the skin was closed with Michel clips.

5.2.5.2 Spinal cord Microinjection of disabled HSV1 vectors

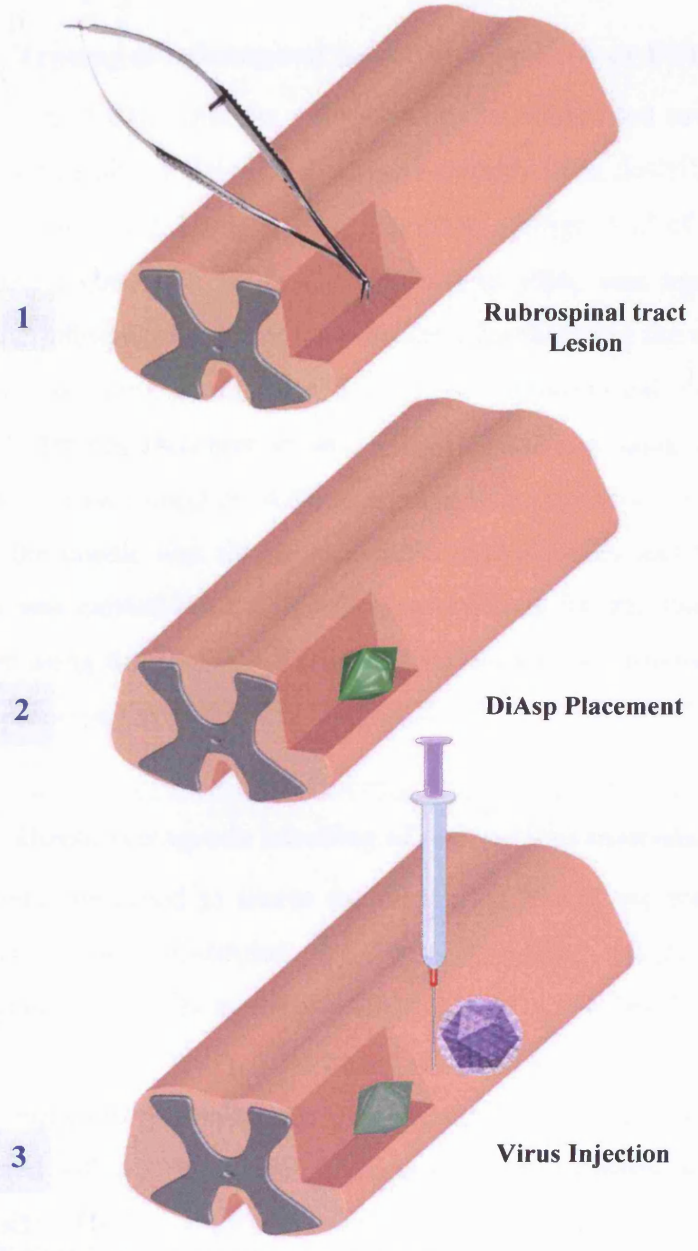
Spinal cords were exposed as described in the previous section and injections were carried out as described in the general methods chapter (Chapter 2). Briefly, a 25 µl Hamilton syringe fitted with a fine needle (outer Ø 300 µm) was used to administer either of the vectors at a 1.5 mm depth into the cord just proximal and distal to the rubrospinal tract lesion site. Viral suspensions were injected at a rate of 0.5 µl/min using a micro-pump. HSV1.pR19CMVrBDNF, HSV1.pR19CMVmCNTF or HSV1.pR19CMVGFP control vectors were delivered in two 2.5 µl aliquots, 5 mm apart and either side of the lesion site, along the rostro-caudal axis of the T4-T6 segments. Ten minutes later the needle was removed, the dura, spine muscles and the overlaying skin was repaired with Michel clips.



Time line (days)	Procedure Performed	Diagram Sign
0	Unilateral rubrospinal tract axotomy at T4 –T6	1
30	Injection: HSV1.pR19CMVrBDNF/mCNTF mixture or HSV1.pR19CMVLacZ, both proximal and distal to the lesion site.	2
45	Injection of the BDA anterograde tracer, close to the contralateral red nucleus	3
60	Termination	

Figure 5.2.5-a: Experimental design: effects of rBDNF & mCNTF on the axotomised RST.

The timeline shown above was followed for the experiments described in section 5.3.3 (page 300). BDA tracer was injected into the red nucleus, contralateral to the location of the lesion site, 15 days prior to termination of the experiment.



Time line (days)	Procedure Performed	Diagram Sign
0	Unilateral rubrospinal tract axotomy at T4 –T6 and	1
	DiAsp crystal insertion.	2
1	Termination of "Non-atrophied" controls.	
30	Injection of HSV1.pR19CMVrBDNF or HSV1.pR19CMVLacZ both proximal and distal to the lesion site.	3
60	Termination	

Figure 5.2.5-b: Experimental design: effects of rBDNF on red nucleus neuron atrophy.

The timeline shown above was followed for the experiments described in section 5.3.4 (page 306). For identifying the neuronal somata of axotomised red nucleus neurons, a single crystal of DiAsp was placed within the rubrospinal tract lesion site, immediately after the lesion was inflicted.

5.2.5.3 Tracing of rubrospinal tract fibres by BDA or DiAsp

Administration of BDA into the rodent brain was carried out using a stereotaxic frame and the details of the procedure have already been described in Chapter 4 as well as in Chapter 2. Using a 25 µl Hamilton syringe, 1 µl of 10% (v/v) BDA tracer (BDA; 10,000MW, Molecular Probes) in PBS, was injected in the red nucleus. The following co-ordinates were used for targeting the rubrospinal tract: rostral-caudal 4.9 mm, medial-lateral 1.4 mm, dorsal-ventral 7.8 mm and at an angle of 5 degrees (Kuchler *et al.*, 2002). Injections were made in 0.2 µl increments over two minutes. After a 10 min wait, to allow for the diffusion of the tracer, the needle was slowly withdrawn and muscles and the skin sutured. Processing was carried out on free-floating sections for the visualisation of the BDA tracer using the HRP-DAB method. Sections were mounted on gelatinised slides and processed as outlined in Chapter 2.

5.2.5.4 DiAsp retrograde labelling of red nucleus neurons

In experiments designed to assess the effects of BDNF on somal atrophy, the BDA tracer was substituted by the lipophilic tracer DiAsp (4-(4-dihexadecylaminostyryl)N-methylpyridium iodide or 4-Di-10-ASP, Molecular Probes Inc., Cat. No: D291). This tracer was applied at the same time that the lesion was inflicted. A small piece of Sterispon (Allen and Handbury, London, UK) saturated with a 20% solution of DiAsp in distilled water, was placed within the lesion site. The pia, dura and overlying musculature was repaired using silk sutures and the skin was closed with Michel clips.

5.2.5.5 SCG10 immunostaining of spinal cord sections

Longitudinal spinal cord sections (40µm) were immunostained for the presence of superior cervical ganglion-10 (SCG10) protein as described previously (Chapter 2). The primary antibody was SCG10 polyclonal antibody (raised in rabbit) obtained from Dr G. Grenningloh (University of Lausanne, Switzerland), used at a dilution of 1:3000. Colour development was carried out using the avidin-biotin complex (ABC) kit as per manufacturer's instructions (Vectastain, Burlingame, CA, USA).

5.2.5.6 Microscopy and quantitation of red nucleus neuron somata size

In experiments designed to explore the effects of vector mediated delivery of rBDNF on atrophy of axotomised red nucleus neurons, it was necessary to employ an automated algorithm-based technique for assessing somal atrophy. Control and treated rat brains were sectioned in the coronal plane at 40 μm and sections thought the midbrain sections were viewed using a Zeiss Axiophot 2 fluorescent microscope (Carl Zeiss Ltd., UK) fitted with a Hamamatsu ORCA 285 digital camera (Hamamatsu Photonics Ltd., UK) and Open-Lab Software (Improvision, University of Warwick, UK).

Software programming was performed in collaboration with Dr G. Campbell (Dept. of Anatomy and Developmental biology, UCL), based on the method described in Kobayashi *et al.* (1997). The DiAsp labelled neurons found contralateral to the lesioned red nucleus on each midbrain section were photographed in both control and treated animals. To quantify the labelled neurons, an automated series of photographs was taken at a magnification of 10x through the entire section thickness at 1μm spacing, using the *Automator* module of the Open-Lab software. Deconvolution of the collected images and subsequent merging produced a single sharp image of the neurons and resulted in the removal of any out-of-focus structures. This single sharp image was similar to a confocal image and showed all labelled neurons in focus throughout the depth of the section (see Figure 5.3.4-a). The surface area of all DiAsp positive neurons in the contralateral lesioned red nucleus in both treated and untreated animals was measured on these single deconvolved images. These measurements were performed using the automated *Measuring Module* function of the Open-Lab software, ensuring that all the somata measured had a visible nucleus. To avoid the possibility of counting the same neuronal cell body from two adjacent sections (e.g. if the microtome blade bisected the cell by passing through its nucleus), a gap of at least one section (40 μm) between measured neurons was allowed, while on average three to five sections were used in order to quantitate the DiAsp positive red nucleus neurons present in each animal. In order to identify which neurons were to be measured, suitable somata were identified based on the intensity of their fluorescence. This was assessed based on two

separate criteria: an upper and then a lower intensity threshold value. In other words the analysis was run twice, once with the upper threshold in place and once with the lower threshold in place (see Figure 5.3.4-b and Figure 5.3.4-c).

Using the *Density-Slice* tool of the Open-Lab software an upper and a lower threshold value for the intensity of fluorescence was set. The upper threshold value was such that it allowed measuring the area of the majority of neurons present on a deconvolved slice, ensuring that all bright areas did not exceed the fluorescent intensity of the brightest neuronal soma. Similarly, the lower threshold value used for the second density slice allowed neuronal cell bodies that display weaker labelling to be included in the measurements (Figure 5.3.4-c). When two labelled cell bodies were in such close proximity to each other that the software failed to differentiate between them, these were measured manually using the *Region Of Interest* (ROI) tool. All profiles less than 8 μm^2 in diameter were filtered out in order to stop any spurious particles from being measured. All measured areas were entered as raw data into the Microsoft® Excel program and statistical analysis was performed as detailed below.

5.3 Results

5.3.1 Functional characterisation of vector HSV1.pR19CMVrBDNF

Prior to proceeding with attempts to insert the rBDNF gene into the viral DNA backbone, it was necessary to confirm its correct insertion into the pGEM5.pR19CMV plasmid. This was done by sequencing the nucleotides on the 3' end of the CMV promoter with a corresponding primer (the primer sequence is outlined in Chapter 2). The plasmid sequence obtained was then compared via BLAST to the rat genome and was found to be compatible (Figure 5.3.1-b). The cloning steps and subsequent recombination events that led to the production of the recombinant vector HSV1.pR19CMVrBDNF are shown schematically in Figure 5.3.1-a. Successful recombinant plaques were selected firstly on the bases of their white phenotype under fluorescence and secondly by molecular screening using Southern blotting analysis (Figure 5.3.1-c, panel **A**). *Eco*RI restriction of the recombinant plaque viral DNA and subsequent probing with an rBDNF specific probe, led to the identification of three plaques which were positive for the presence of a ≈1130bp band corresponding to the full length rBDNF gene (Maisonpierre *et al.*, 1991). This band corresponds to the one obtained via an *Eco*RI restriction of pSP72/rBDNF and pGEM5.pR19CMVrBDNF plasmid DNA which were used as positive controls. No bands were identified when the similarly digested HSV1.pR19CMVGFP vector backbone (negative control) was exposed to the same rBDNF probe.

Following the identification of successful recombinants, the HSV1.pR19CMVrBDNF vector was propagated to a concentrated stock and its ability to transduce non-complementing cells was assessed *in vitro*. The procedure of sample collection is similar to that described elsewhere (Chapter 4, section 4.3.1). $1x10^6$ BHK cells were infected (MOI=1) with either HSV1.pR19CMVGFP (n=2) or HSV1.pR19CMVrBDNF (n=8 in total). Supernatants were collected only at 8 hours in the case of HSV1.pR19CMVGFP (control) and at 2, 4, 6 and 8 hour time points in the case of HSV1.pR19CMVrBDNF infected cells (n=2 per time point). In addition, the cell lysate from the 8-hour time point (n=1) was collected and analysed along with the conditioned media (supernatants) by Western blotting analysis.

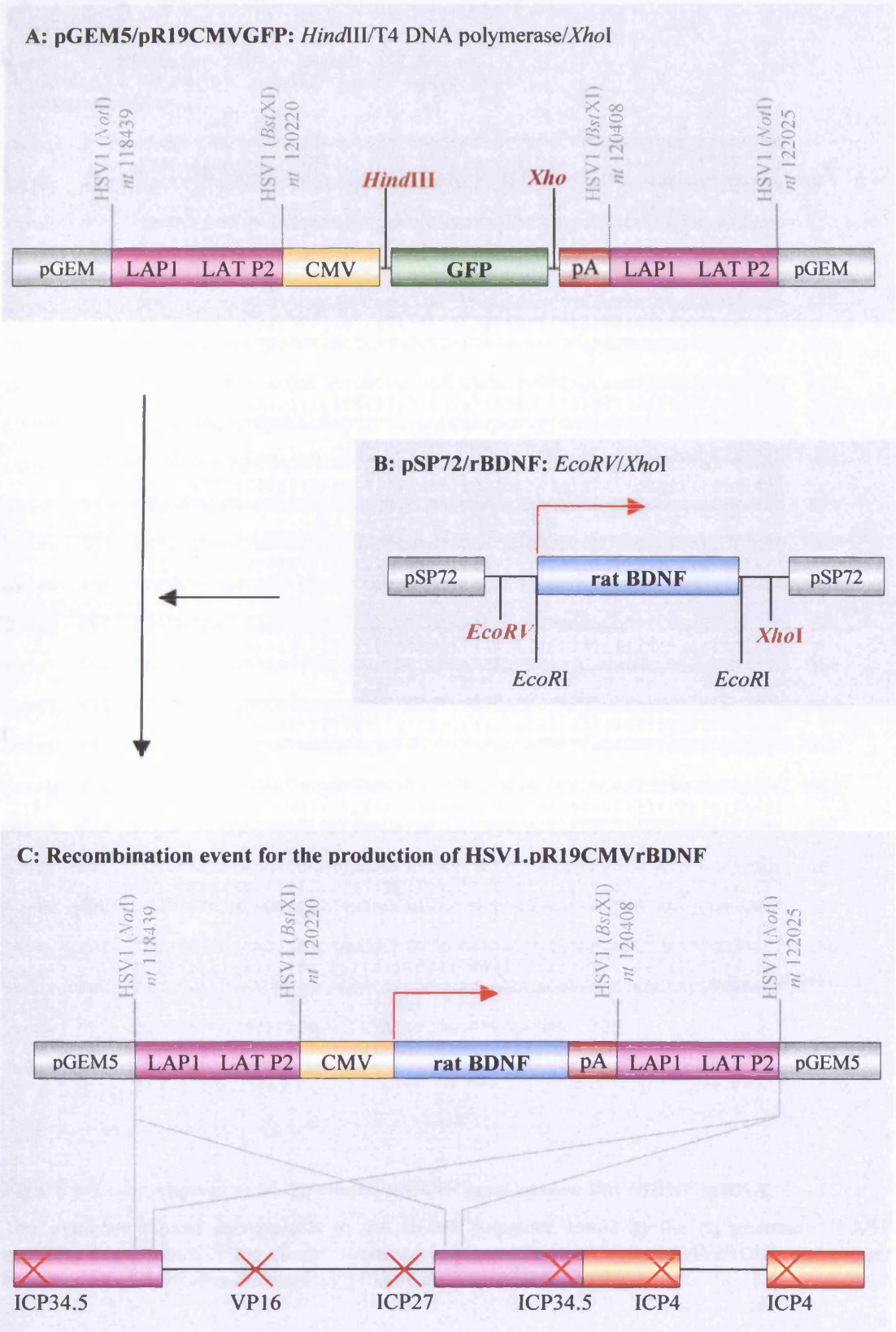


Figure 5.3.1-a: Construction of vector HSV1.pR19CMVrBDNF.

```
>gi|45544888|gb|AY559248.1| Rattus norvegicus brain-derived neurotrophic factor
(BDNF) mRNA, complete cds, alternatively spliced
Length=3794

 Score = 1293 bits (700),  Expect = 0.0
 Identities = 700/700 (100%), Gaps = 0/700 (0%)
 Strand=Plus/Plus

Query  1    CAGTTCCACCAGGTGAGAAGAGTGATGACCATCCTTTTCCTTACTATGGTTATTTCATAC  60
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  140  CAGTTCCACCAGGTGAGAAGAGTGATGACCATCCTTTTCCTTACTATGGTTATTTCATAC  199

Query  61   TTCGGTTGCATGAAGGCTGCGCCCATGAAAGAAGCAAACGTCCACGGACAAGGCAACTTG  120
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
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Query  121  GCCTACCCAGCTGTGCGGACCCATGGGACTCTGGAGAGCGTGAATGGGCCCAGGGCAGGT  180
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  260  GCCTACCCAGCTGTGCGGACCCATGGGACTCTGGAGAGCGTGAATGGGCCCAGGGCAGGT  319

Query  181  TCGAGAGGTCTGACGACGACGTCCCTGGCTGACACTTTTGAGCACGTGATCGAAGAGCTG  240
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  320  TCGAGAGGTCTGACGACGACGTCCCTGGCTGACACTTTTGAGCACGTGATCGAAGAGCTG  379

Query  241  CTGGATGAGGACCAGAAGGTTCGGCCCAACGAAGAAAACCATAAGGACGCGGACTTGTAC  300
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  380  CTGGATGAGGACCAGAAGGTTCGGCCCAACGAAGAAAACCATAAGGACGCGGACTTGTAC  439

Query  301  ACTTCCCGGGTGATGCTCAGCAGTCAAGTGCCTTTGGAGCCTCCTCTGCTCTTTCTGCTG  360
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  440  ACTTCCCGGGTGATGCTCAGCAGTCAAGTGCCTTTGGAGCCTCCTCTGCTCTTTCTGCTG  499

Query  361  GAGGAATACAAAAATTACCTGGATGCCGCAAACATGTCTATGAGGGTTCGGCGCCACTCC  420
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Sbjct  500  GAGGAATACAAAAATTACCTGGATGCCGCAAACATGTCTATGAGGGTTCGGCGCCACTCC  559

Query  421  GACCCCGCCCGCCGTGGGGAGCTGAGCGTGTGTGACAGTATTAGCGAGTGGGTCACAGCG  480
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Sbjct  560  GACCCCGCCCGCCGTGGGGAGCTGAGCGTGTGTGACAGTATTAGCGAGTGGGTCACAGCG  619

Query  481  GCAGATAAAAAGACTGCAGTGGACATGTCCGGTGGGACGGTCACAGTCCTGGAGAAAGTC  540
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Query  541  CCGGTATCAAAAGGCCAACTGAAGCAATATTTCTACGAGACCAAGTGTAATCCCATGGGT  600
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Sbjct  680  CCGGTATCAAAAGGCCAACTGAAGCAATATTTCTACGAGACCAAGTGTAATCCCATGGGT  739

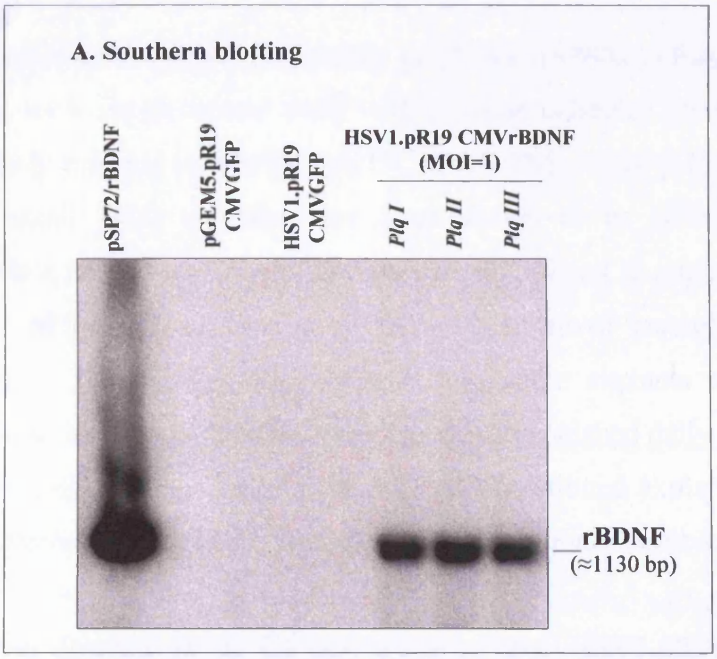
Query  601  TACACGAAGGAAGGCTGCAGGGGCATAGACAAAAGGCACTGGAACTCGCAATGCCGAACT  660
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  740  TACACGAAGGAAGGCTGCAGGGGCATAGACAAAAGGCACTGGAACTCGCAATGCCGAACT  799

Query  661  ACCCAATCGTATGTTCGGGCCCTTACTATGGATAGCAAAA  700
            ||||||||||||||||||||||||||||||||||||||||
Sbjct  800  ACCCAATCGTATGTTCGGGCCCTTACTATGGATAGCAAAA  839
```

Figure 5.3.1-b: Alignment of the cloned rBDNF gene against the rBDNF mRNA.

The sequence cloned corresponds to the BDNF sequence found in the rat genome. BLAST sequence comparison of the cloned sequence in plasmid pGEM5/pR19CMVrBDNF and the rat sequence from the *Rattus Norvegicus* genome corresponding to rBDNF.

As can be seen in Figure 5.3.1-c (panel **Bi**) all conditioned medium time points are positive for the presence of a ≈14.5 kDa band that according to the literature corresponds to the fully processed, mature and potentially bioactive rBDNF (Mowla *et al.*, 2001). The ≈32 kDa band corresponding to the pro-form of rBDNF and is attributed to the glycosylation that increases the stability of the pro form (Mowla *et al.*, 2001). According to Mowla and colleagues (2001), some pro-BDNF can be released extracellularly and is biologically active since it has been shown to mediate TrkB phosphorylation. Comparison between the amount of pro-rBDNF present in the supernatant (Figure 5.3.1-c, panel **Bi**) and in the cell lysate (Figure 5.3.1-c, panel **Bii**) for the 8-hour time point sample (n=1) suggests that the immature form is mostly intracellular but its presence extracellularly is increased with increasing time points. The increasing presence of the pro-rBDNF ≈32 kDa band in the medium at later time points may be attributed to two factors. Firstly, the inevitability of cell lysis, which is a direct result of not only the high MOI used but also of the fact that the infected monolayer has been deprived of serum for a significant amount of time. Secondly, it could be a reflection of the efficiency of the CMV promoter in transcript production that results in large amounts of the pro-rBDNF protein. This accumulation may overwhelm the downstream processing mechanism in the trans-Golgi network, responsible for the N-glycosylation and N-terminal cleavage that leads to the production of the mature BDNF form (Mowla *et al.*, 2001). The intracellular 28 kDa rBDNF band noted in cell lysates could correspond to an intermediate of the pro-rBDNF form, which has also been reported by other researchers. Even though not an obligatory intermediate to the conversion of pro-BDNF to its mature counterpart, the 28 kDa intermediate has been previously reported to occur due to alternative processing mechanisms that are still under investigation (Mowla *et al.*, 2001). As it is apparent from the Western Blot analysis described above, the HSV1.pR19CMVrBDNF vector can deliver and drive the expression of rBDNF in a non-complementing cell line. Moreover the vector produces the full-length transcript capable of being processed from the full-length 32 kDa pro form and its 28 kDa intermediate, to the 14.5 kDa mature rBDNF form. Significantly, the mature form is secreted from the transduced cell and could therefore affect adjacent cells.



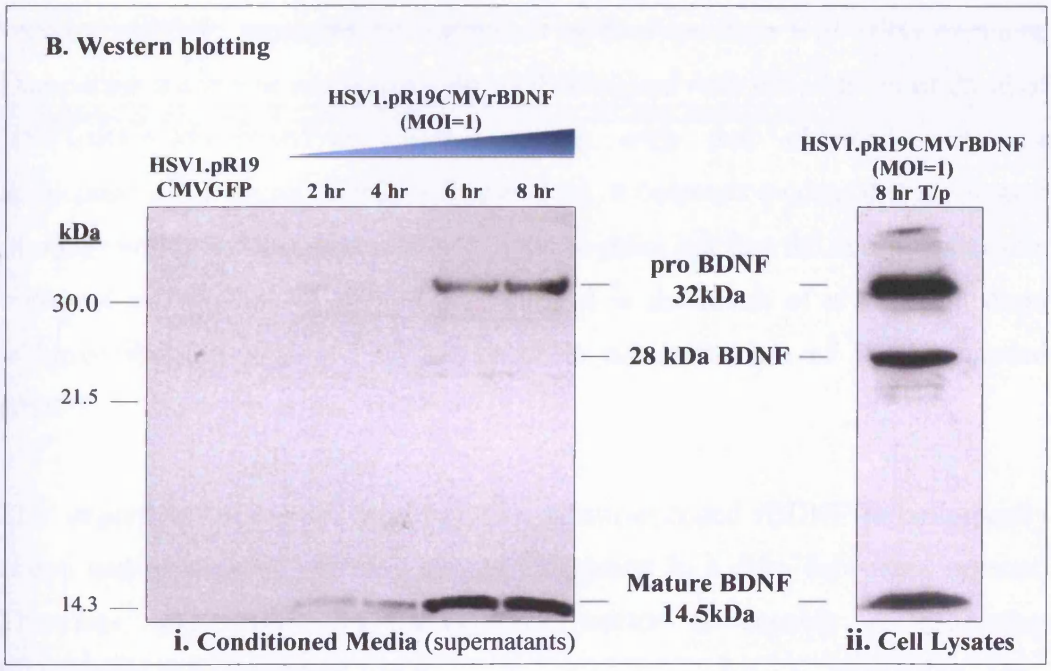


Figure 5.3.1-c: Functional characterisation of vector HSV1.pR19CMVrBDNF.

A. Southern blotting on three purified recombinant plaques of vector HSV1.pR19CMVrBDNF. pSP72/rBDNF and pGEM5/pR19CMVrBDNF plasmids were used as positive controls while HSV1.pR19CMVGFP viral DNA was used as a negative control. The single ≈1130 bp band recognised by the rBDNF probe used (pSP72/rBDNF, *EcoR*I digest) corresponds to the rBDNF gene (Maisonpierre *et al.*, 1991) and is present in the recombinant vector genome.

B. Western blotting using a rabbit polyclonal anti-rat BDNF capable of recognising the 32 kDa pro-BDNF form, 28 kDa intermediate form and the fully processed 14 kDa mature form. **i:** Conditioned media from $1x10^6$ BHK cells, infected (MOI=1) with either HSV1.pR19CMVGFP (negative control) or HSV1.pR19CMVrBDNF. Secretion of the 14.5 kDa mature rBDNF form in the medium increases with longer time points (2, 4, 6 and 8 hours). **ii:** Cell lysate from the 8-hour time point reveals the presence of the pro-rBDNF processing intermediate as well as accumulation of some mature rBDNF that has not been secreted possibly due to the fact that the amount of protein exceeds the processing or secretory capacity of the cell.

In order to prove the bioactivity of vector produced rBDNF, collagen embedded E14 explants were supplemented daily with medium collected from $1x10^6$ BHK cells, previously infected with HSV1.pR19CMVrBDNF vector (n=3, MOI=1). At E14, some small DRG neurons have been shown to be BDNF responsive (Barakat-Walter, 1996), and could therefore be stimulated to extend neurites in the presence of biologically active rBDNF. 10 ng/ml of recombinant human BDNF (rhBDNF) (n=2) was directly added to some explants to serve as a positive control. Vector-conditioned medium was replenished daily following the removal of cellular debris. Positive control and conditioned explants were fixed and immunostained with a TUJ1 neuron specific anti-tubulin antibody.

Figure 5.3.1-d (panels **B** & **C**) demonstrates that HSV1.pR19CMVrBDNF encoded rBDNF, promotes the extension of neurites from E14 DRG explants. Comparing the extent of neurite outgrowth achieved with the addition of 25 µl of HSV1pR19CMVrBDNF-conditioned media with that obtained with the application of 10 ng/ml of rhBDNF (panel **A**), it becomes evident that the degree of outgrowth is similar. It is also important to point out that the neurite extension observed in the case of the positive control is the result of a single medium administration as opposed to the multiple administration of vector-encoded rBDNF.

This experiment therefore confirms that vector-encoded rBDNF is biologically active and capable of inducing axonal elongation in a dose dependent manner. Therefore, the HSV1.pR19CMVrBDNF vector is capable of producing biologically active rBDNF.

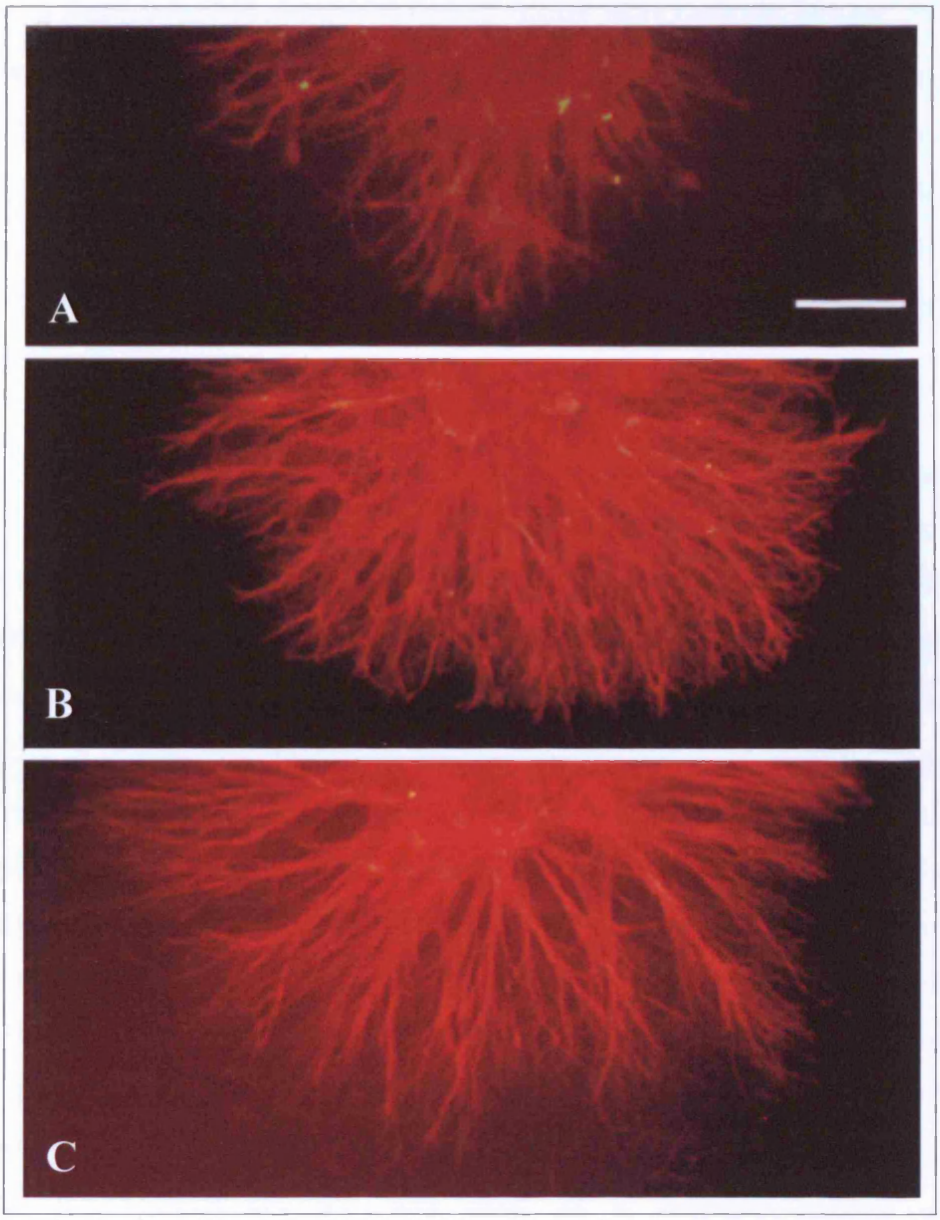


Figure 5.3.1-d: Biological assay for vector encoded rBDNF on E14 rat DRGs.

TUJ1 immunolabelled neurites of collagen embedded E14 DRGs treated with **A**, **B** or **C** media. Note that collagen embedded explants were supplemented with 10 ng/ml of NGF (2.5S) which acts as a survival factor. All images show TUJ1 immunostaining on explants.

A. Positive control E14 explants supplemented with 10 ng/ml of recombinant human BDNF, display a distinctive neurite extension. (Maximal activity is reported at 50 ng/ml, R&D systems protocol).

B. Explants supplemented with 25 µl of media from $1x10^6$ BHK cells infected with HSV1.pR19CMVrBDNF cells at an MOI of 1 display a dense halo of thick neurite bundles.

C. The halo of neurites appears denser and the individual fibres extend further when 100µl of conditioned media is added to the explants demonstrating the dose dependent effect of vector-expressed rBDNF on neurite elongation.

Scale Bar: 500µm, applies to all figures.

5.3.2 Functional characterisation of vector HSV1.pR19CMVmCNTF

Construction of vector HSV1.pR19CMVmCNTF is diagrammatically outlined in Figure 5.3.1-a, while the overall procedure has been already described for two other vectors. As explained in section 5.2.2, the mCNTF gene utilised in this set of experiments is a chimera that contains the murine NGF-β subunit secretory signal (Sendtner *et al.*, 1992). This enables the protein to be exported from the transduced cell.

Correct insertion of the mCNTF gene was confirmed by restriction digest analysis and sequencing from the pGEM5/pR19CMVmCNTF plasmid, using a primers specific to the CMV promoter. The resulting sequence was then entered into a BLAST search and homologous sequences were identified automatically. As shown in Figure 5.3.2-b, the sequence contains the NGF-β secretory signal and the mCNTF gene in succession and on the 3' end of the CMV promoter. Following recombination of the pGEM5/pR19CMVmCNTF plasmid with an HSV1.pR19CMVGFP backbone, successful recombinant populations were identified, initially based on their white phenotype and subsequently by Southern blotting analysis. Two recombinant plaques of white phenotype were digested by *Hind*III/*Xmn*I and probed with a mCNTF probe. They were both found to have a ≈951bp fragment corresponding to the 5' end of mCNTF, which includes the NGF secretory sequence (Figure 5.3.2-c, panel **A**). Viral DNA from the vector backbone HSV1.pR19CMVGFP was used as a negative control while pmCNTFchim5 and pGEM5/pR19CMVmCNTF plasmid DNAs were used as positive controls. The new vector was termed HSV1.pR19CMVmCNTF.

The ability of this vector to transduce BHK cells thus enabling them to produce mCNTF, was assessed by Western blotting. In addition, the ability of this protein to produce a biological effect was assessed by a cell proliferation assay on a CNTF responsive cell line. For sample collection, $1x10^6$ BHK cells were infected (MOI=1) with either HSV1.pR19CMVGFP (n=12) or HSV1.pR19CMVmCNTF (n=12). Supernatants from HSV1.pR19CMVGFP (negative control) or HSV1.pR19CMVmCNTF infected cells were collected at a single time point of 6 hours. It was thought that at this time point there would be potentially enough

bioactive mCNTF present while at the same time the amount of denatured protein would be relatively low. This would be ideal in terms of inducing a biological response. All samples were cleared of cellular debris prior to analysis.

The partially reducing Western blotting analysis performed, shown in Figure 5.3.2-c (panel **B**), clearly shows the presence of two distinct bands: a low molecular weight band of ≈23 kDa, representing the monomeric form of the protein and a high molecular weight band of ≈46 kDa which represents the mCNTF dimer. The simultaneous detection of these forms was possible because the SDS-PAGE gel used for the analysis partially disrupts disulfide bridges. Rat as well as human CNTF has been shown to exist primarily as a monomer but significant dimer formation occurs at protein concentrations exceeding 40μM (McDonald *et al.*, 1995; Narhi *et al.*, 1997) perhaps reflecting an artificial association. However, it should be made clear that the presence of dimers in the murine version of CNTF has not been directly examined. Even though the role of dimeric-CNTF is not clear, it has been proposed that it plays a role in the efficient storage of CNTF within Schwann cells in peripheral neural tissue (McDonald *et al.*, 1995). Whether dimeric CNTF is capable of CNTF-Rα receptor activation is also not clear. However, McDonald and colleagues (1995) suggest that the fact that CNTF binds to its receptor with a 1:1 stoichiometry in solution (Panayotatos *et al.*, 1994) makes it unlikely that dimers can bind and activate the receptor. The significance of dimer formation with the secreted form of CNTF is currently unclear but it might not be related to the ability of this neurotrophic factor to activate its receptor.

Overall, this experiment demonstrates that the HSV1.pR19CMVmCNTF vector can transduce a non-complementing cell line such as BHK cells and drive the production of a secretable form of mCNTF that is capable of dimer formation. Significantly, the transduced cells can secret the monomeric form of CNTF which is thought to be responsible for CNTF-Rα receptor activation (Ip *et al.*, 1993; Panayotatos *et al.*, 1994).

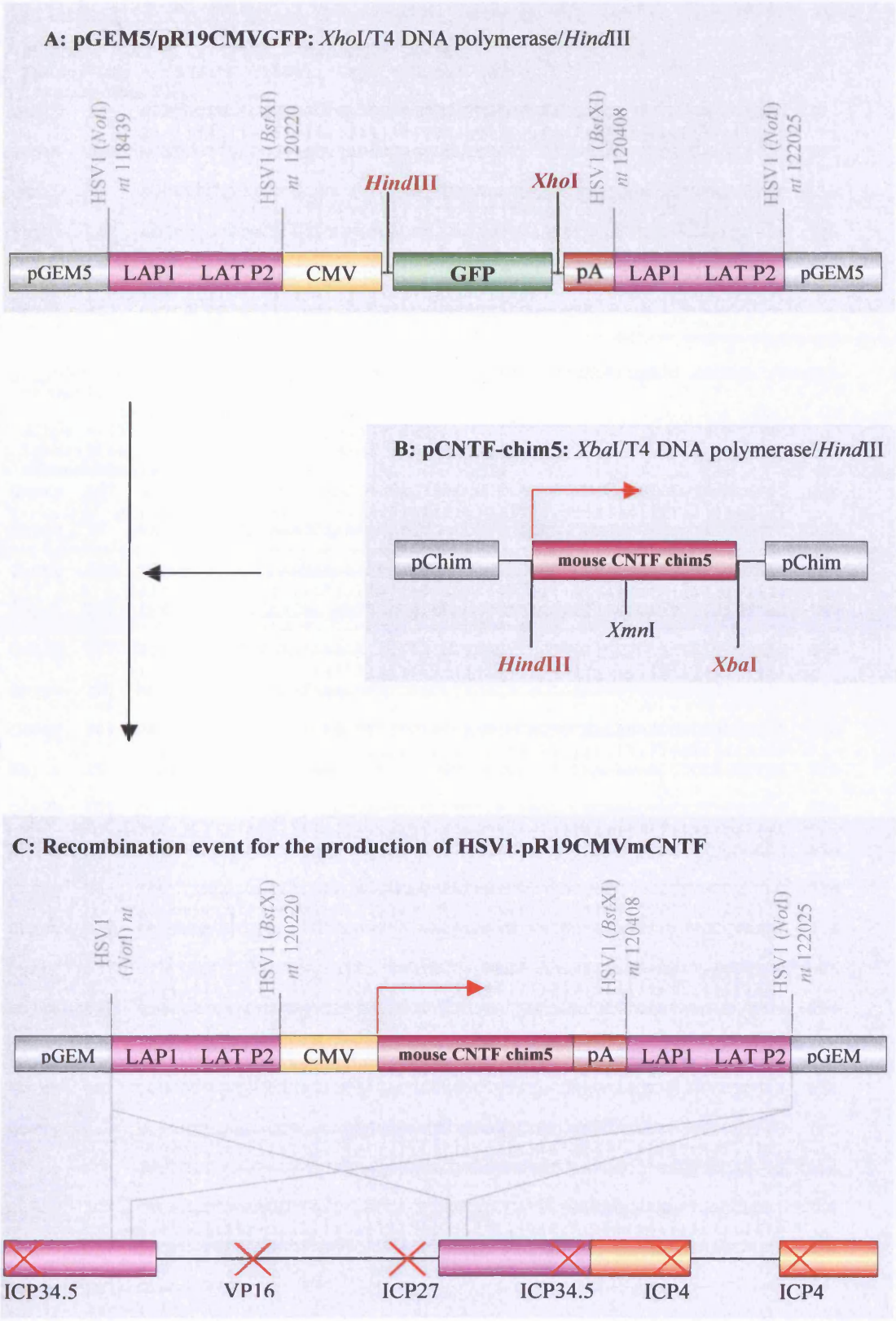


Figure 5.3.2-a: Construction of vector HSV1.pR19CMVmCNTF.

```
gi|7305312|ref|NM_013609.1| Mus musculus nerve growth factor, beta (Ngfb), mRNA
           Length=1181
 Score =  302 bits (157),  Expect = 1e-78
 Identities = 157/157 (100%), Gaps = 0/157 (0%)
 Strand=Plus/Plus
Query  11   GCATGCTGGACCCAAGCTCACCTCAGTGTCTGGGCCCAATAAAGGTTTTGCCAAGGACGC  70
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Query  71   AGCTTTCTATACTGGCCGCAGTGAGGTGCATAGCGTAATGTCCATGTTGTTCTACACTCT  130
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Query  131  GATCACTGCGTTTTTGATCGGCGTACAGGCAGAACCG  167
            |||||||||||||||||||||||||||||||||||||
Sbjct  322  GATCACTGCGTTTTTGATCGGCGTACAGGCAGAACCG  358


gi|16507246|ref|NM_053007.1| Mus musculus ciliary neurotrophic factor (Cntf),
transcript variant 2, mRNA
           Length=1893
 Score = 1161 bits (604),  Expect = 0.0
 Identities = 604/604 (100%), Gaps = 0/604 (0%)
 Strand=Plus/Plus
Query  167  GATGGCTTTCGCAGAGCAAACACCTCTGACCCTTCACCGCCGGGACCTCTGTAGCCGTTC  226
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Query  227  TATCTGGCTAGCAAGGAAGATTCGTTCAGACCTGACTGCTCTTATGGAATCTTATGTAAA  286
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Query  467  AACTGAAGGTGACTTCCATCAGGCAATACATACTCTTATGCTCCAAGTTTCTGCCTTTGC  526
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
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Query  527  CTACCAGCTAGAGGAGTTAATGGTGCTTCTGGAACAGAAGATCCCTGAAAATGAGGCTGA  586
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
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Query  587  TGGGATGCCTGCCACAGTTGGAGATGGTGGTCTCTTTGAGAAGAAGCTATGGGGCTTGAA  646
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
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Query  647  GGTCCTTCAAGAGCTCTCACAGTGGACTGTGAGGTCTATCCATGACCTTCGTGTCATTTC  706
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Query  707  TTCTCATCAGATGGGAATCTCAGCACTTGAGAGCCATTATGGGGCCAAAGATAAGCAGAT  766
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  617  TTCTCATCAGATGGGAATCTCAGCACTTGAGAGCCATTATGGGGCCAAAGATAAGCAGAT  676

Query  767  GTAG  770
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Sbjct  677  GTAG  680
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Figure 5.3.2-b: Alignment of the *mCNTFchim5* gene with BLAST identified sequences.

The mCNTF gene sequence (Sendtner *et al.*, 1992) cloned into the pGEM5/pR19CMV vector was matched via a BLAST search to the secretory portion of NGF-β subunit (Query nt 11 to 167) and the mCNTF (Query nt 167 to 770).

In order to demonstrate that HSV1.pR19CMVmCNTF-transduced cells are capable of secreting biologically active protein, a proliferation assay was employed based on the TF-1.CN5α.1 cell line. Medium collected from HSV1.pR19CMVmCNTF-infected BHK cells was compared to medium collected from HSV1.pR19CMVGFP infected cells (negative control) for its ability to promote TF-1.CN5α.1 cell division. Control or mCNTF conditioned medium (n=3) were assessed at 5, 25 and 50μl aliquots (n=3 per aliquot per time point tested) for their ability to induce proliferation in $3x10^4$ TF-1.CN5α.1 cells. Cell numbers were assessed in triplicate. Recombinant human CNTF (rhCNTF) was used as a positive control at a final concentration of 2 ng/ml. Figure 5.3.2-c (panel **C**) demonstrates that addition of mCNTF vector-conditioned medium, induces the TF-1.CN5α.1 cells to divide in a dose-responsive manner. The effect observed with the addition of 50 μl of HSV1.pR19CMVmCNTF conditioned medium at the 6-day time point is higher than that observed with the addition of rhCNTF at the same time point. Six days after the addition of HSV1.pR19CMVmCNTF conditioned medium in $3x10^4$ TF-1.CN5α.1 cells the number of cells had increased to $11.8x10^4$ cells (± 0.37) (Mean ± Standard error of the mean). On the contrary there were $3.17x10^4$ (± 0.41) and $8.32x10^4$ (±0.33) cells counted when the cell line was supplemented with HSV1.pR19CMVGFP conditioned medium or rhCNTF (positive control) respectively. Untreated cell numbers were recorded at $3.63x10^4$ cells (± 0.34) at the 6-day time point. As it can be seen from the graph in Figure 5.3.2-c (panel **C**), the number of TF-1.CN5α.1 cells present at the 6-day time point following the addition of 2 ng/ml of rhCNTF is significantly lower than the numbers present when the cells are stimulated with the addition of 50 μl of HSV1.pR19CMVmCNTF vector conditioned medium. This is probably due to the fact that the vector conditioned medium was replenished daily while the more expensive rhCNTF was not. Addition of HSV1.pR19CMVGFP conditioned medium failed to promote cell division, re-enforcing the fact that cell division is specific to the mCNTF vector encoded protein and not a non specific effect of the backbone vector itself. This experiment demonstrates that non-complementing cells, infected by vector HSV1.pR19CMVmCNTF, are capable of synthesising and secreting biologically active mCNTF.

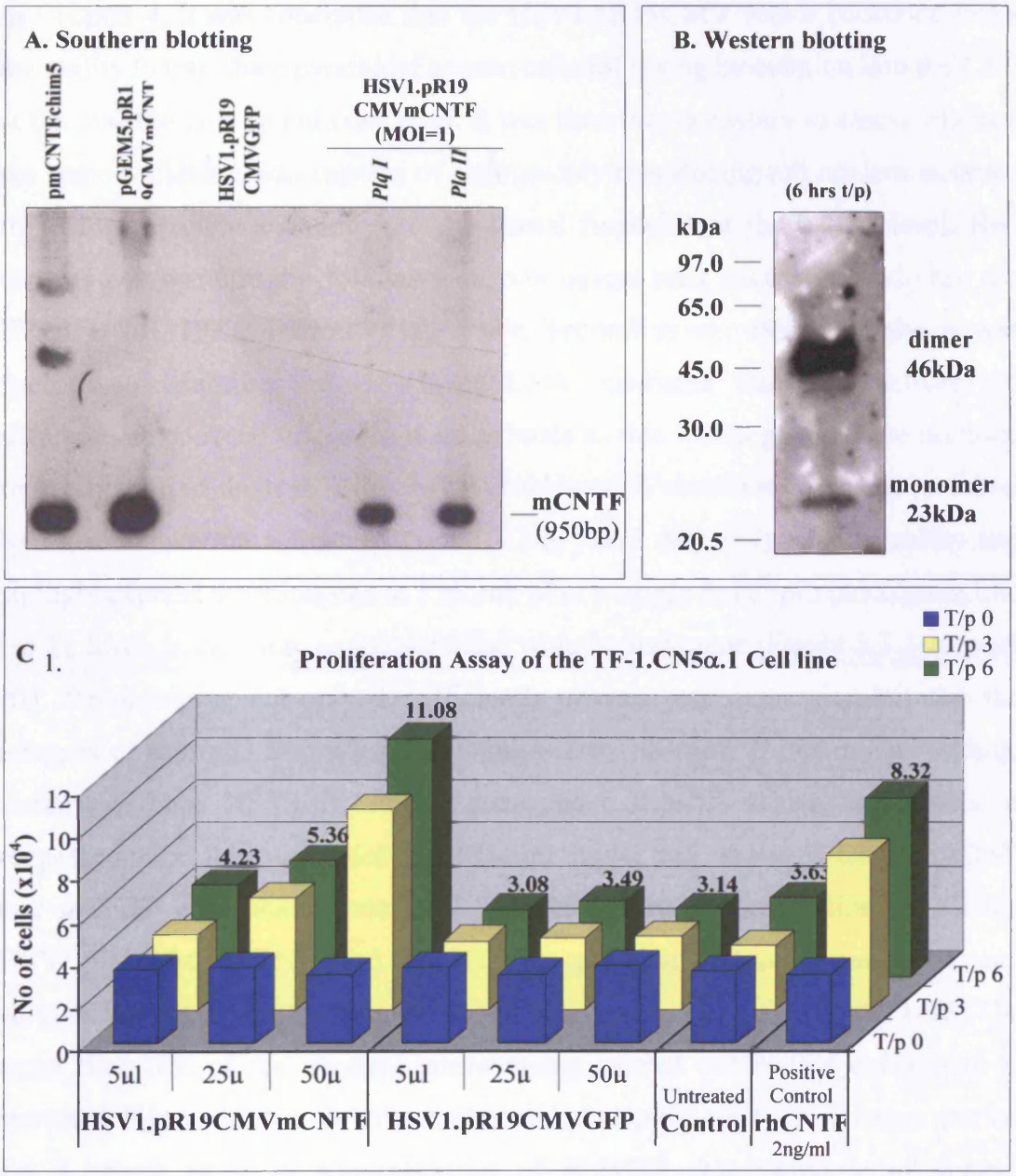


Figure 5.3.2-c: Functional characterisation of vector HSV1.pR19CMVmCNTF.

A. Southern blotting on two purified recombinant plaques of vector HSV1.pR19CMVmCNTF. pmCNTFchim5 and pGEM5/pR19CMVmCNTF plasmids were used as positive controls while HSV1.pR19CMVGFP viral DNA was used as a negative control. The single 950bp mCNTF band recognised by the mCNTF probe (*Hind*III/*Xmn*I digest) is present in the viral genome.

B. Western blotting (with minimally reducing conditions) analysis of conditioned media from $1x10^6$ BHK cells previously infected at an MOI of 1 with HSV1.pR19CMVmCNTF and probed with a goat polyclonal ant-rat CNTF.

C. Proliferation assay on the TF-1.CN5α.1 cell line. TF-1.CN5α.1 cells divide when bathed in virally conditioned, mCNTF containing media. Cell division is dose-dependent and resembles the response noted when media was supplemented with 2 ng/ml of rhCNTF (positive control). No such response is elicited with media obtained from HSV1.pR19CMVGFP infected BHK cells (negative control).

5.3.3 Effect of vector rBDNF & mCNTF on lesioned rubrospinal axons

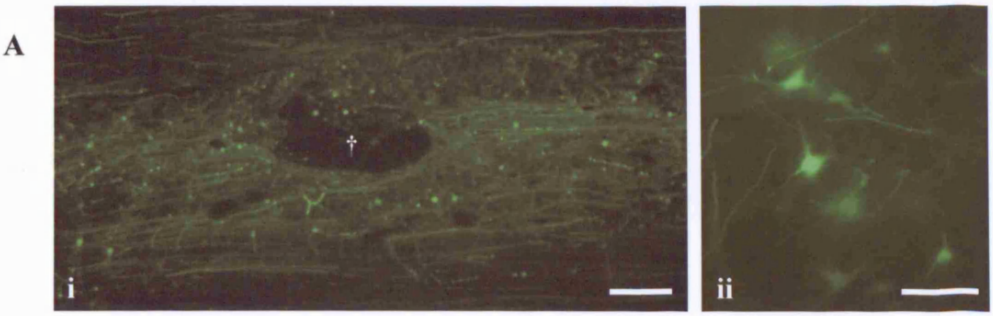
In Chapter 4, it was concluded that the HSV1.pR19CMV vector backbone lacks the ability to transduce pyramidal neuron cells following inoculation into the CST at the mid-thoracic spinal cord level. It was therefore necessary to assess whether the same backbone was capable of retrogradely transducing red nucleus neurons following direct inoculation into the lateral funiculus at the T4-T6 level. Red nucleus neurons atrophy following an rubrospinal tract axotomy but do not die (Egan *et al.*, 1977; Barron *et al.*, 1989; Tetzlaff *et al.*, 1991). Firstly, it was decided to examine the HSV1.pR19CMV mediated transgene delivery in uninjured as opposed to pre-lesioned animals as this would give a more accurate representation of the true ability of this backbone to sustain transgene expression. Red nucleus neuron somata (Figure 5.3.3-a, panel **Aii**, n=4) appear healthy and strongly express the transgene at 1 month after a single $5x10^6$ pfu inoculation into the T6 level. In addition, axons are filled with the transgene (Figure 5.3.3-a, panel **Ai**), demonstrating not only the efficiently of transgene expression but also the integrity of neuronal machinery following vector infection. Based on this finding, vector backbone HSV1.pR19CMV presented a suitable vehicle for studies of regeneration on the rubrospinal tract lesion model and was used for the rBDNF and mCNTF expressing constructs. The ability of a combination of vectors HSV1.pR19CMVrBDNF and mCNTF to promote axonal regeneration and survival was assessed in animals that had been previously lesioned at T4-T6. The exact time line of the surgical interventions carried out in this experiment is shown in Figure 5.2.5-a. Briefly, unilateral rubrospinal tract lesions were carried out 1 month prior to administration of a $5x10^6$ equal mixture of vectors HSV1.pR19CMVrBDNF and mCNTF, delivered both proximal and distal to the lesion site. Two months post-lesion, 40 μm horizontal spinal cord sections were reacted for BDA, the anterograde tracer injected close to the red nucleus ≈2 weeks prior to termination, in order to label any potentially regenerating rubrospinal tract axons. In rBDNF/mCNTF treated animals (Figure 5.3.3-a, panel **Bi**, n=11), a number of BDA positive axons were found within as well as proximal to the lesion site (▼). They appear to follow an irregular route around the lesion site with only a few axons present within the lesion site.

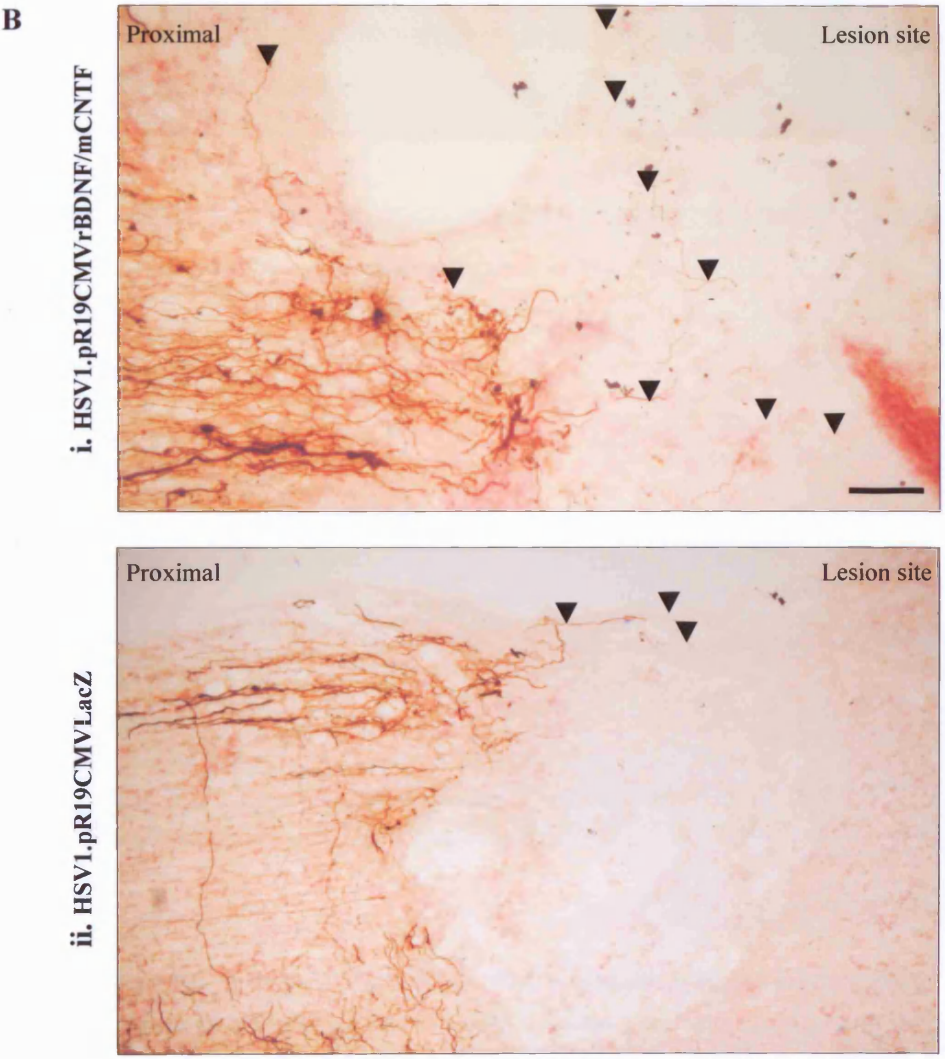
Figure 5.3.3-a: HSV1.pR19CMVrBDNF/mCNTF minimally promotes regeneration.

A. HSV1.pR19CMVGFP vector was delivered at a single injection site (†) into the T4-T6 level of the spinal cord, targeting rubrospinal tract axons. At one month post-injection, strong GFP labelling can be seen in rubrospinal tract axons (coursing left to right) at the spinal cord level (**Ai**) and also on the transduced red nucleus neurons (**Aii**).

B. HSV1.pR19CMVrBDNF/mCNTF vector mixture (**Bi**) or HSV1.pR19CMVLacZ vector (**Bii**) were injected both proximal and distal to the site of a unilateral rubrospinal tract lesion the animals had received 1 month previously. BDA labelled axons (▼) can be seen sprouting into the lesion site of the HSV1.pR19CMVrBDNF/mCNTF treated animal (**Bi**) with rather fewer sprouts apparent in the HSV1.pR19CMVLacZ treated animal (**Bii**).

Scale bars: **Ai**=200μm; **Aii**=100μm; **Bi**=60μm, applies to **Bii**.


A
i
ii


B
i. HSV1.pR19CMVrBDNF/mCNTF
Proximal
Lesion site
ii. HSV1.pR19CMVLacZ
Proximal
Lesion site

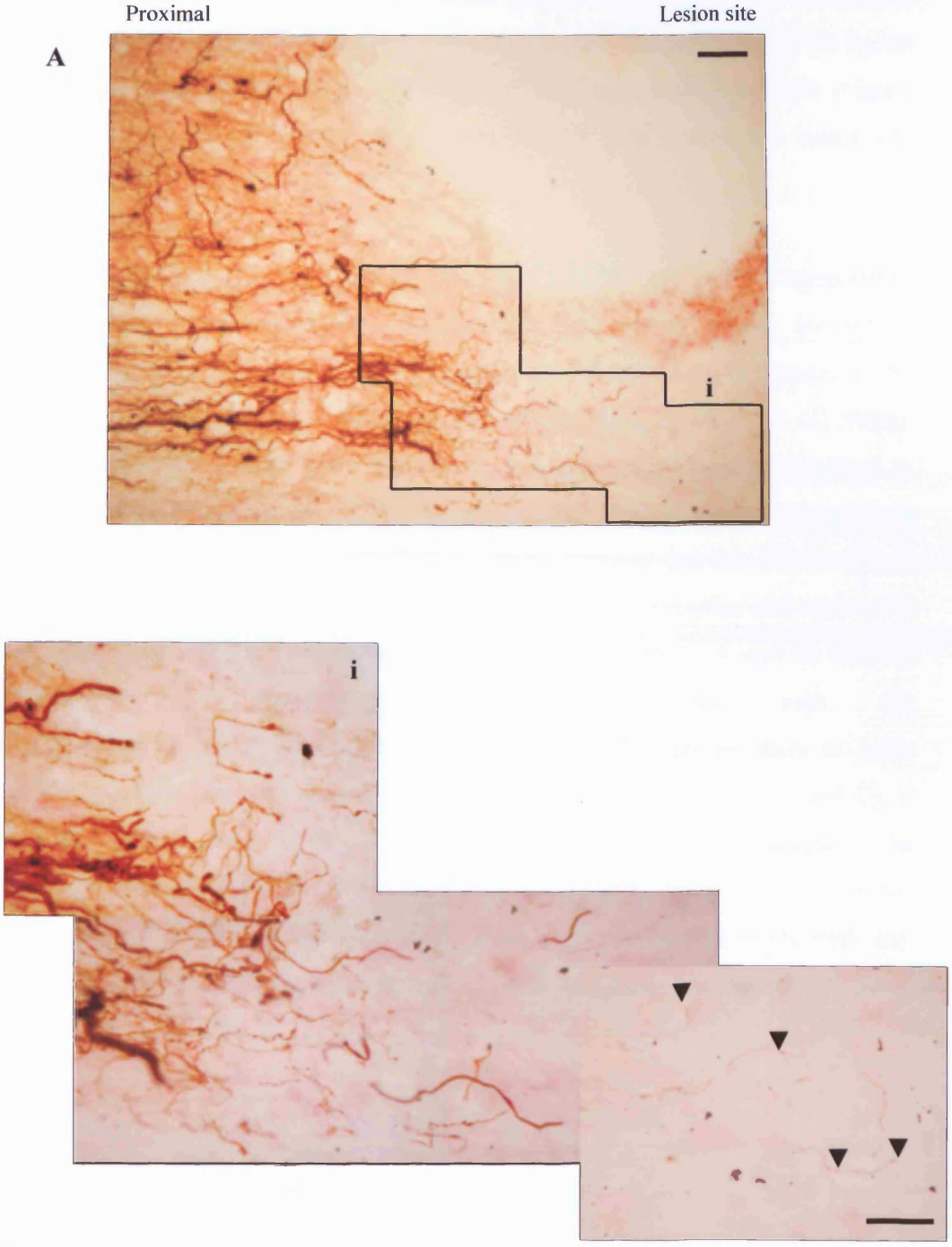


Figure 5.3.3-b: HSV1.pR19CMVrBDNF/mCNTF minimally promotes regeneration.

BDA labelled axons (▼) in an additional animal from the same series shown in Fig. 5.3.3-a (**B**) injected with a mixture of HSV1.pR19CMVrBDNF/mCNTF vectors. **A.** a number of BDA labelled axons in box **i** can be found around the lesion site (upper right). The enlargement of box **i** (panel beneath) shows that their route is irregular. Note that the actual lesion site has been lost during processing. Scale bars: **A**=50μm; enlarged inset **i**=30μm.

In another rBDNF/mCNTF treated animal (Figure 5.3.3-b), the few axons seen within the lesion site follow an irregular route around the perimeter of the lesion site rather than through it. In HSV1.pR19CMVLacZ treated animals (Figure 5.3.3-a, panel **Bii**, n=4), very few axons can be seen around the lesion site boundaries.

Even though vector mediated administration of rBDNF and mCNTF appeared to only slightly affect rubrospinal tract axonal regeneration it was decided to examine whether this modest effect is accompanied by the up-regulation of the growth-associated protein neuron specific protein SCG10 (Stein *et al.*, 1988). SCG10 has been shown to accumulate in axonal terminals and is involved in elongation (McNeill *et al.*, 1999) by modulating the interaction of neuronal microtubules (Riederer *et al.*, 1997). It has been proposed that this feature allows it to play a role as a synaptic plasticity regulator in the developing and adult CNS (McNeill *et al.*, 1999). As shown in Figure 5.3.3-c (panels **A** and **B**) SCG10 expression is upregulated in animals treated with the HSV1.pR19CMVrBDNF/mCNTF vector mixture. Even though there is some SCG10 positive labelling in HSV1.pR19CMVLacZ control animals (panel **C**), it appears to be less prominent than that observed in HSV1.pR19CMVrBDNF/mCNTF treated animals (panel **B**). However, this up-regulation of SCG10 in treated animals does not appear to correlate with any anatomically significant regeneration, as is evident in previous sections.

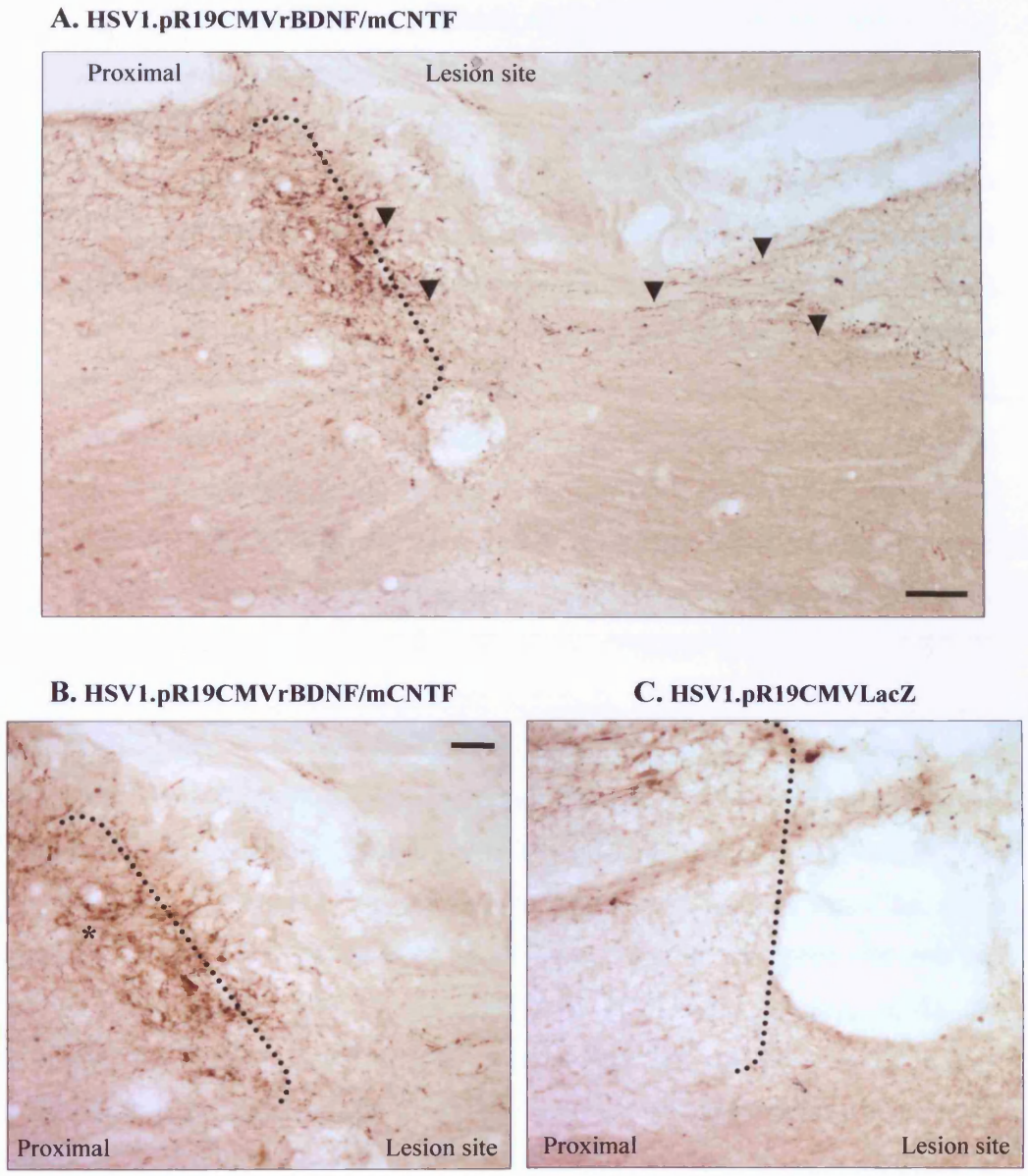


Figure 5.3.3-c: rBDNF/mCNTF delivery & SCG10 expression on rubrospinal tract axons.

A. SCG10 immunolabelled axons in a sagittal section through the lesion site in a spinal cord of an animal receiving the HSV1.pR19CMVrBDNF/mCNTF mixture (**B** shows the lesion site at higher magnification). Note that the majority of axons appear to congregate on the proximal side of the lesion site (delineated by the dotted line). Also, there are more axons present within and distally (arrowheads) to the lesion site of the HSV1.pR19CMVrBDNF/mCNTF treated animal than there are in the control, HSV1.pR19CMVLacZ vector treated animal (**C**).

Scale bars: **A**=200μm; **B**=100μm, applies to **C**.

5.3.4 Effect of rBDNF on cell body atrophy of red nucleus somata

Previous reports suggested that exogenous BDNF is able to reverse the atrophy of red nucleus neurons that follows axotomy when delivered close to their cell bodies (Kobayashi *et al.*, 1997; Novikova *et al.*, 2000; Kwon *et al.*, 2002). In this series of experiments red nucleus neurons were transduced with vector HSV1.pR19CMVrBDNF in an attempt to reproduce this effect after chronic injury of the rubrospinal tract in the rat. The ability of the HSV1.pR19CMV backbone to retrogradely transduce these neurons (Figure 5.3.3-a, panel **A**) presented the opportunity to target BDNF to the cell bodies, while avoiding the invasiveness of a direct injection into the red nucleus. A schematic representation of the timeline regarding the surgical interventions that were performed in this experiment is shown in page Figure 5.2.5-b. Briefly, the rubrospinal tract was transected in control and treated animals via a unilateral laminectomy at T4-T6 (section 5.2.5.1, page 265). Lesion site integrity was maintained by placing a small piece of Sterispon® soaked in DiAsp within the lesion site. This allowed the subsequent visualisation of axotomised red nucleus neurons and prevented the formation of a dense scar. Twenty-four hours following axotomy and DiAsp administration, one animal (Rat A) was terminated and used as a healthy, non-atrophied control. Four weeks later, in all other animals, the lesion site was re-exposed and $5x10^6$ pfu of HSV1.pR19CMVrBDNF (treated, n=5) or HSV1.pR19CMVLacZ (control, n=2) vector was delivered proximally to the lesion site. The animal numbers given here reflect those animals finally included in the statistical analysis of somal areas. Originally, 15 animals were allocated in the HSV1.pR19CMVrBDNF group, 6 animals in the HSV1.pR19CMVLacZ group and 3 animals in the non-atrophied negative control groups. Animals that died or not displaying <u>any</u> DiAsp labelled red nucleus neurons were excluded. After 30 days, treated and control animals were killed and 40μm coronal sections of the magnocellular part of the caudal, contralateral red nucleus were examined under fluorescence for the presence of DiAsp positive neurons (Figure 5.3.4-c, panel **A**). DiAsp positive neurons contralateral to the lesion were photographed and images were then deconvolved so that all positive neurons were brought to focus in a single plane (Figure 5.3.4-a).

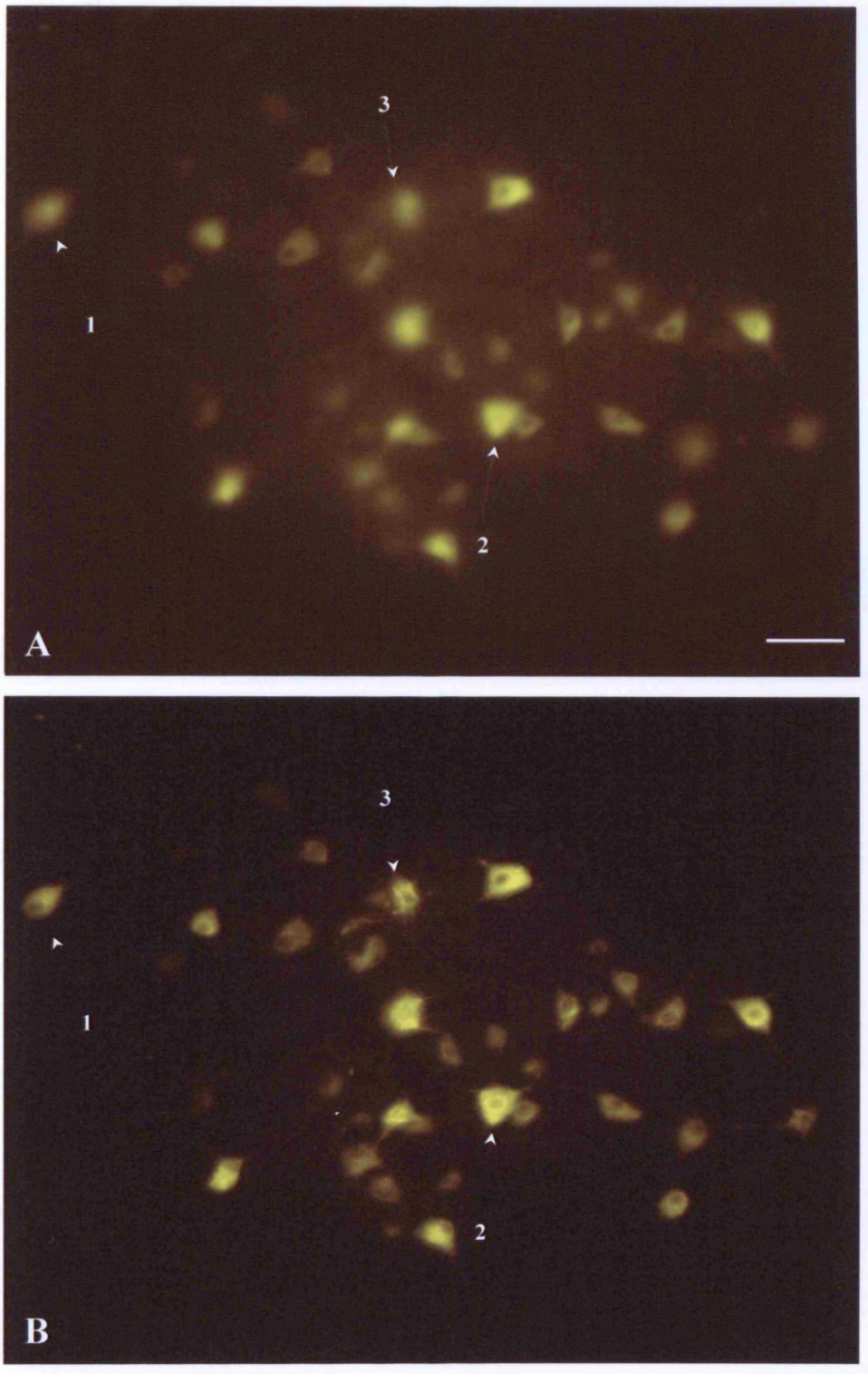


Figure 5.3.4-a: Deconvolved images of DiAsp labelled red nucleus neurons.

A. A wet 40μm section, photographed at a single plane of focus, appears to have few DiAsp labelled neurons in the red nucleus contralateral to a labelled rubrospinal tract lesion. **B.** Merging a series of deconvolved images of the same neurons shown in **A** yields a focused image which shows more neurons throughout the depth of the section. Arrows mark the same neurons out of focus in a single non-deconvolved image (**A**) and in focus in the merged, deconvolved image (**B**). Scale bar = 250μm.

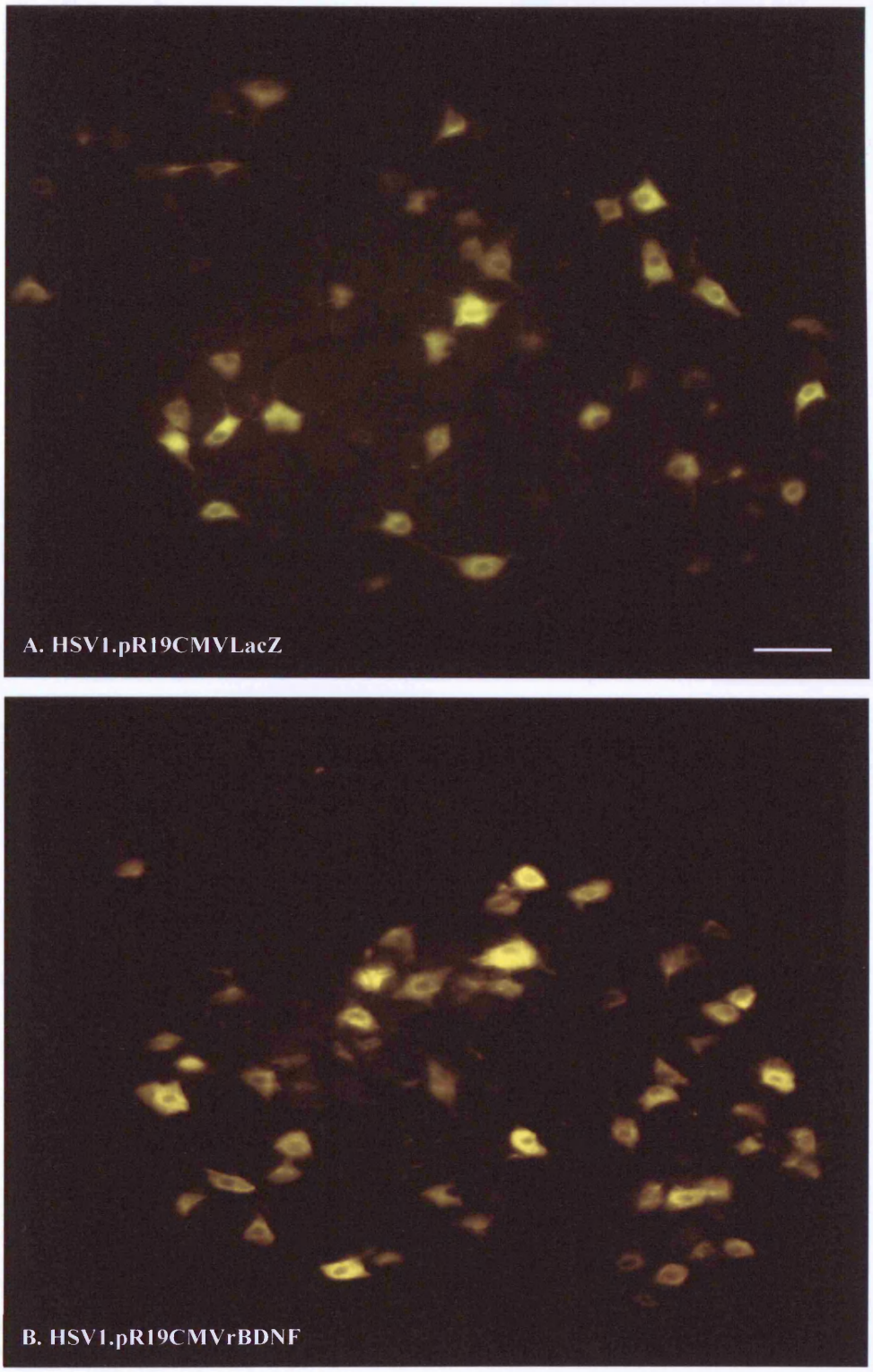


Figure 5.3.4-b: DiAsp labelling in control and HSV1.pR19CMVrBDNF treated animals.

Red nucleus neurons retrogradely labelled with DiAsp viewed in wet, unmounted sections. Animals received a rubrospinal tract injury 4 weeks prior to the injection of either HSV1.pR19CMVLacZ (**A**) or HSV1.pR19CMVrBDNF (**B**) proximal to the rubrospinal tract lesion. Note that there are more labelled neurons in the BDNF vector treated animal (**B**) compared with the LacZ treated animal (**A**). Scale bar: **A**=250μm, applies to **B**.

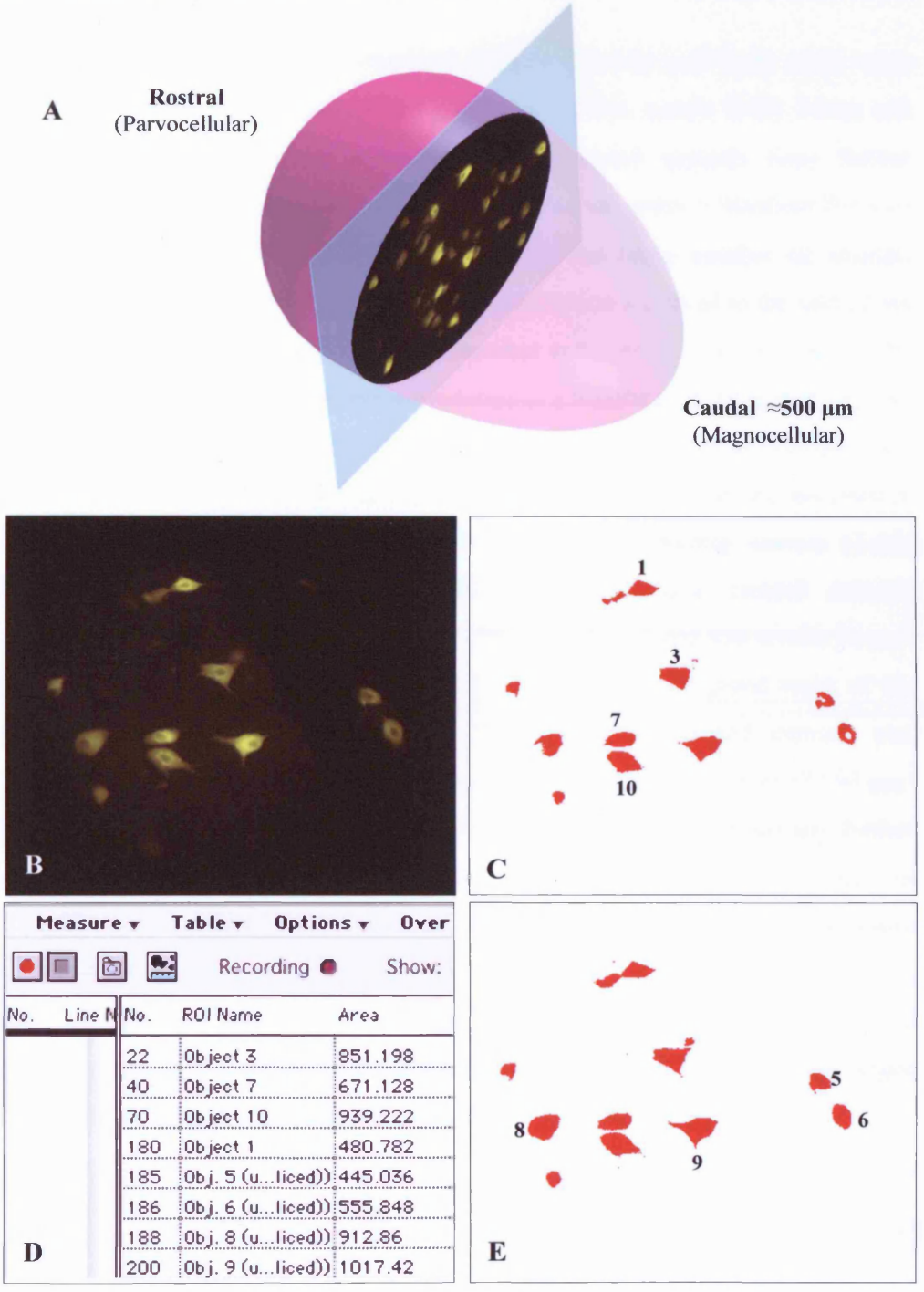


Figure 5.3.4-c: Software based acquisition of red nucleus somal areas.

Deconvolved sections from the magnocellular part of the red nucleus were used. A schematic representation of their location is shown in **A** (adapted from Ruitenberg *et al.* 2004). Neurons were photographed in wet, unmounted sections and a merged deconvolved image (**B**) was used to generate 2 sets of density slices (**C**, **E**). Based on these and using the Open-Lab software it was possible to calculate actual somal areas (listed in **D**). The threshold for the density slice in **C** was chosen because it adequately filled the outlines of the somata of neurons 1, 3, 7 and 10 without overlapping their perimeters. Because this threshold was too low for neurons 5, 6, 8 and 9 their somata were not adequately filled (shown in **E**) a second threshold was taken and density slice in **D** obtained.

From the deconvolved images obtained (Figure 5.3.4-b) cell body areas were calculated using the Open-Lab software (Figure 5.3.4-c, panels **B-D**). Mean cell body areas (μm^2) measured in treated and untreated animals were further analysed and shown as Mean red nucleus neuron somal areas ± Standard Error of the Mean (SEM) (Table 5.3-a and b). Despite the large number of animals initially included in this study, only a small proportion survived to the end of the experiment. This was unfortunate but somewhat anticipated especially due to the repeated surgery these animals had to undergo in a relatively short period of time. Mean red nucleus neuron cell body areas for control and treated animals were measured at two months following axotomy and one month following injection of HSV1.pR19CMVLacZ or HSV1.pR19CMVrBDNF expressing vectors (Table 5.3-a). Red nucleus neurons from HSV1.pR19CMVLacZ treated animals displayed a mean somal area of 425.30±72.08 μm^2 (Rat 1) and 430.63±20.76 μm^2 (Rat 2) giving a range of 353.22 to 497.38 μm^2 (Rat 1). The grand mean of the red nucleus somal areas from HSV1.pR19CMVrBDNF treated animals was found to be 538.25±34.72 μm^2, which represents a range of 503.53 to 572.97 μm^2 (Rats 3-6, n=4, Table 5.3-b). Even though the sample sizes precluded any further statistical evaluation, overall, the mean red nucleus cell body areas in HSV1.pR19CMVrBDNF treated animals (Rats 3-6) appear to be somewhat larger that those of HSV1.pR19CMVLacZ controls (Rats 1&2). It is important to emphasise that even though this result may be suggestive of a beneficial effect of BDNF on red nuclei neurons following a lesion of the RST, clearly larger samples would be needed before firm conclusions can be drawn.

	Animal Group	Rat No	Mean Cell Body Area (μm^2) (Mean ± SEM)	Counts
Controls	Immediate lesion	A	700.94 ± 29.71	49
	HSV1.pR19CMVLacZ Control (n=2)	1	425.30 ± 72.08	5
		2	430.63 ± 20.76	39
Treated	HSV1.pR19CMVrBDNF Treated (n=4)	3	485.22 ± 17.55	85
		4	471.56 ± 22.68	44
		5	593.73 ± 21.62	62
		6	602.51 ± 17.26	169

Table 5.3-a: Mean cell body after LacZ or HSV1.pR19CMVrBDNF treatment.

Mean red nucleus cell body areas μm^2 ± Standard Error of the Mean (SEM).

Animal Group		Mean Somal Area (μm^2) (Mean ± SEM)
HSV1.pR19CMVLacZ (Controls)	Rat 1	425.30 ± 72.08
	Rat 2	430.63 ± 20.76
HSV1.pR19CMVrBDNF Treated (n=4) Rats 3 - 6		*538.25 ± 34.72

Table 5.3-b: Mean red nucleus somal areas in control versus BDNF-treated animals.

While the mean somal size for the two control animals (Rat 1 and Rat 2) lie below and outside the range of the treated group (n=4), the sample sizes precluded statistical evaluation. Nevertheless, this may suggest a beneficial effect of BDNF on red nuclei neurons following lesioning of the RST. However, larger samples would be needed before any conclusions can be drawn.

***Note:** The mean somal area value shown for the HSV1.pR19CMVrBDNF treated animals represents the Grand mean value obtained from the individual mean red nucleus neuron somal areas of each of the four treated animals (Rats 3-6, detailed in Table 5.3-a).

5.4 Discussion

The rubrospinal tract has been the focus of many recent studies of spinal cord regeneration. The relative ease of surgical manipulation of this tract has been exploited in these *in vivo* experiments to investigate both the capabilities and limitations of neurotrophin expressing, highly disabled HSV1.pR19CMV-based vectors in preventing atrophy of red nucleus neurons and promoting regeneration of their axons. Two new neurotrophin-expressing vectors were constructed: HSV1.pR19CMVrBDNF and HSV1.pR19CMVmCNTF. These were based on the well characterised, highly disabled 1764/ICP27$^-$/ICP4$^-$/RL1$^-$/pR19CMV backbone (Lilley *et al.*, 2001b). This backbone can retrogradely transduce red nucleus somata and support transgene expression for at least one month following a single inoculation into the adult rat rubrospinal tract, at the thoracic level (Figure 5.3.3-a). BDNF and CNTF were selected based on previous *in vivo* evidence that these factors have the potential to prevent red nucleus somal atrophy and support axonal regeneration (reviewed in sections 5.1.4.1 and 5.1.4.2). Each of these vectors was shown *in vitro* to be able to stably transduce a non-complementing cell line and support the expression and secretion of biologically active rBDNF (section 5.3.1) or mCNTF (section 5.3.2).

Following the characterisation of these vectors it was decided to examine their effects on axonal regeneration and on atrophy of red nucleus neuronal cell bodies. In the first series of experiments (section 5.3.3), an equal mixture of vectors HSV1.pR19CMVrBDNF and HSV1.pR19CMVmCNTF was inoculated both proximally and distally to the site of a 1-month-old thoracic lesion, in an attempt to promote rubrospinal tract axonal regeneration. Two months following the application of the rBDNF/mCNTF vector mixture there was no evidence of any significant axonal regeneration despite a modest increase in SCG10 expression in HSV1.pR19CMVrBDNF/HSV1.pR19CMVmCNTF treated animals. Although disappointing the lack of regeneration observed in these experiments was in agreement with other reports of lack of axonal regeneration following the local infusion of BDNF at the spinal cord level. In particular, Kwon *et al.* (2004) report that delivering BDNF at a cervical rubrospinal tract injury site 2 months after lesion, fails to prevent the red nucleus somal atrophy that sets in over the

subsequent weeks and does not lead to any axonal regeneration (Kwon *et al.*, 2004). The notable lack of rubrospinal tract axonal regeneration could be due to the fact that there is a time-limited window of opportunity for influencing axonal regeneration via BDNF administration (Kwon *et al.*, 2004). Axons of red nucleus neurons, unlike their somata, cease to express full length TrkB following axotomy and would therefore be unresponsive to locally supplied BDNF (Liebl *et al.*, 2001; Kwon *et al.*, 2004). In our case however it was anticipated that the use of the HSV1.pR19CMV based vectors would circumvent this problem since it would allow the injured neuron to synthesise *de novo* the transgene BDNF that would be made available at the cell body level which maintains full-length expression of TrkB for at least one year post-injury (Kwon *et al.*, 2002). Alternatively, the failure in axonal elongation could be attributed to the by now widely accepted notion that any meaningful axonal regeneration would require additional molecules counteracting lesion site inhibitory signals (Fawcett and Asher, 1999).

As in the case of the CST experiments described in Chapter 4, the approach followed in these studies may also have been too ambitious. Thoracic level lesions, as employed in this series of experiments do not provoke the same degree of upregulation of growth associated proteins, such as GAP43 and α-Tubulin, as cervical lesions do (Tetzlaff *et al.*, 1991; Tetzlaff *et al.*, 1994; Fernandes *et al.*, 1999). It may therefore have been more appropriate to focus our rBDNF and mCNTF, vector mediated regenerative attempts on the cervical rather than thoracic rubrospinal tract lesion model. Alternatively it might also have been more reasonable to transduce intact red nucleus neurons first and then inflict a thoracic or cervical rubrospinal tract lesion a few days later i.e. once transgene expression was established. Admittedly, this approach may not have properly addressed the true potential of these vectors and the neurotrophic factors they encode to promote regeneration in a realistic, chronic rubrospinal tract lesion setting. It would however have allowed us to examine whether or not rBDNF/mCNTF vector mediated delivery is capable of influencing the extent of atrophy or examine any possible effects on the pattern of growth associated protein up-regulation.

The second line of experimentation focused on the question of whether vector mediated rBDNF delivery could prevent the atrophy of axotomised red nucleus neurons. Previous reports have suggested that BDNF can prevent atrophy when it is administered to the cell bodies of red nucleus neurons acutely after injury and promote the upregulation of GAP43 and Tα1 tubulin (Kobayashi *et al.*, 1997; Kwon *et al.*, 2002). As shown in Table 5.3-b, administration of the rBDNF-expressing vector one month following a T4-T6 rubrospinal tract lesion does appear to have some beneficial effect in red nucleus cell body atrophy. The mean soma area was 425.30±72.08 μm^2 (Rat 1) and 430.63±20.76 μm^2 (Rat 2) for HSV1.pR19CMVLacZ controls (n=2) and 538.25±34.72 μm^2 for HSV1.pR19CMVrBDNF treated animals (n=4). The above results suggest that treatment of axotomised rubrospinal tract neurons with vector HSV1.pR19CMVrBDNF at one month following a thoracic level lesion may have a beneficial effect in reversing the red nucleus neuronal atrophy that ensues an rubrospinal tract lesion. This result would appear to be in line with previous reports that document the ability of rBDNF to reverse red nucleus atrophy when delivered by either an infusion pump (Kobayashi *et al.*, 1997; Novikova *et al.*, 2000; Kwon *et al.*, 2002) or an AAV vector (Ruitenberg *et al.*, 2004) close to the red nucleus somata.

Despite the fact that the results presented here represent true differences, due to the technical complexity of these experiments the comparisons were based on only two HSV1.pR19CMVLacZ controls and four HSV1.pR19CMVrBDNF treated animals. It therefore follows that, though encouraging, the results of this study should be interpreted conservatively. This is mainly because they are based on a comparison with only two negative control animals that received the HSV1.pR19CMVLacZ vector. At best the results described here can only serve as an indication that HSV1.pR19CMV vector mediated delivery of rBDNF may hold promise as a way of transducing lesioned red nucleus neurons with potentially beneficial effects on neuronal atrophy. Several attempts were made to repeat this series of experiments but due to persisting technically difficulties it was not possible to reproduce this response within the time frame of this study.

In a single animal, chorea-like movements were observed within 5 days following rBDNF vector administration. At the time this was noted only in Rat 6 and affected the upper limb, ipsilateral to the lesion site. It is possible that this was simply due to an unrelated event such as an intracranial haemorrhage. Alternatively, it could be due the fact that vector mediated rBDNF expression occurs in areas closely associated with structures that regulate fine movement and may cause aberrant sprouting in these areas. The onset of these effects would also coincide with the vector mediated production of sufficient amounts of rBDNF from transduced red nucleus neuronal somata (Lilley *et al.*, 2001b). For example the substantia nigra pars-compacta is closely associated with the red nucleus, which is potentially overproducing rBDNF due to vector-mediated transduction. This observation is in line with other reports of similar viral vector studies. Ruitenberg and colleagues report that some of the animals that had received an AAV-BDNF expressing vector via direct injection into the red nucleus exhibited rotation-like behaviour (Ruitenberg *et al.*, 2002; Ruitenberg *et al.*, 2004). In addition, BDNF infusion (Altar *et al.*, 1992) or substantia nigra transduction via an AAV-BDNF vector (Klein *et al.*, 1999) apparently led to increased rotational behaviour and locomotor activity. These reports support the proposed role of BDNF as a modifier of basal ganglia output by affecting dopaminergic neuron output and potentiating the effects of glutamaergic neurotransmission (Arenas *et al.*, 1996). Long term BDNF over-expression is associated with increased vulnerability of these neurons to ischemic damage (Gustafsson *et al.*, 2003).

In addition to the explanations above, there are certain features specific to animal 6 that may also help explain why this occurred in this particular animal. The number of DiAsp labelled red nucleus neurons found in animal 6 (169 counts) is nearly double the number found in the other three similarly treated animals (Table 5.3-a). One possibility is that vector inoculation was more efficient in this animal, leading to the transduction of a higher number of axotomised red nucleus neurons. On the other hand, the larger number could simply indicate that DiAsp labelling was more efficient in this animal. Unfortunately it was not possible to co-localise DiAsp and rBDNF expression, as subsequent processing would have abolished the DiAsp signal.

SUMMARY

It was originally hypothesised that if vectors HSV1.pR19CMVrBDNF and HSV1.pR19CMVmCNTF are capable of transducing injured RST neurons to *de novo* synthesise biologically active rBDNF and mCNTF, then these constructs may lead to a more favoured regenerative response in injured RST neurons. In terms of regeneration within the context of the injured rubrospinal tract, the combination of HSV1.pR19CMVrBDNF and HSV1.pR19CMVmCNTF did not lead to any meaningful axonal regeneration in the chronically injured rubrospinal tract, despite inducing upregulation of SCG10 and enhancing axonal sprouting. In addition, HSV1.pR19CMVrBDNF transduction of chronically injured red nucleus neurons does appear to somewhat limit the extent of red nucleus somal atrophy even though this should be considered a preliminary experiment that should be repeated with larger animal groups.

Overall, the experiments described in this chapter lead to the conclusion that the highly disabled HSV1.pR19CMV based vectors can serve as a powerful platform to evaluate potentially therapeutic molecules such as BDNF and CNTF. Further improvements in the surgical procedures employed would in theory allow for a more detailed evaluation of potentially therapeutic molecules and the extent of their impact within more closely defined and reproducible experimental parameters.

CHAPTER 6

VECTOR - MEDIATED NEUROTROPHIC FACTOR DELIVERY IN OPTIC NERVE REGENERATION

6.1 Introduction

Due to its well-defined anatomical structure and ease of accessibility, the rodent optic tract has been intensively used in attempts at promoting neuronal survival, plasticity and regeneration of injured retinal ganglion cells (RGCs). This valuable animal model enables researchers to explore somal and axonal responses to injury separately. RGCs of adult mammals, unlike their fish and amphibian counterparts (Reier and Webster, 1974; Murray and Edwards, 1982), die in response to injury and fail to successfully regenerate (Ramon Y Cajal, 1928). Intraorbital transection of the adult rat optic nerve results in an 85% loss of RGCs within two weeks (Villegas-Perez *et al.*, 1993). Following an optic nerve crush, a very small number of RGC axons are able to spontaneously regenerate through the lesion site (Campbell *et al.*, 1999). This inability of RGCs to regenerate is most likely the result of a combination of inhibitory molecules present in the injured CNS milieu, lack of trophic factor support and changes in the growth capacity of adult mammalian neurons. Cell death and regenerative failure can potentially be altered by manipulating the injured axon environment (Levin and Gordon, 2002). Lesioned adult RGCs are responsive to a variety of neurotrophic and growth factors *in vivo*. Intraoccular administrations of BDNF, CNTF, GDNF or FGF have been shown to be beneficial in enhancing survival of axotomised RGCs and in supporting axonal regeneration (Sievers *et al.*, 1987; Mey and Thanos, 1993; Mansour-Robaey *et al.*, 1994; Sawai *et al.*, 1996; Cui *et al.*, 1999). Vectors expressing each of the above neurotrophins were used in various combinations to promote axonal regeneration in the crushed optic nerve paradigm. The ability of these constructs to deliver marker genes in the retina via retrograde transport is characterised in the adult albino rat visual system.

6.1.1 Anatomy of the rat visual system

The gross anatomy of the rat eye is demonstrated in Figure 6.1.1-a (Fischer *et al.*, 2000). For the purposes of this study, emphasis is placed on the microscopic anatomy of the retina and the projections of retinal ganglion neurons to their corresponding targets in the rat visual centres.

6.1.1.1 Anatomy of the rat retina

The rodent retina is composed of five types of neuronal cells: photoreceptors, bipolar, horizontal, amacrine and ganglion cells and two types of glial cells: Müller glia and astrocytes. Retinal cells exhibit a laminar organisation with layers referenced to the middle of the eye-ball (Figure 6.1.1-b). This laminar organisation involves three layers of cell bodies separated by two plexiform layers of axons and dendrites. The inner-most layer is the ganglion cell layer, containing the somata of RGCs, amacrine cells and astrocytes. The next layer is the inner nuclear layer, containing the cell bodies of bipolar, horizontal and amacrine cells as well as the cell bodies of Müller glia. Next follows the outer nuclear layer containing photoreceptor cell bodies. In addition to these cellular layers of the retina, there are also several distinct layers of neuropil. In between the ganglion cell layer and the inner nuclear layer is the inner plexiform layer containing the synaptic contacts between RGC dendrites and axons of bipolar and amacrine cells. Similarly between the outer nuclear layer and inner nuclear layer is the outer plexiform layer where photoreceptors synapse with bipolar and amacrine cells (Bear *et al.*, 2001). The outer-most layer is the layer of photoreceptor outer segments containing the light responsive elements of the retina. Müller glia (Chao *et al.*, 1997) extend their processes towards both the ganglion cell layer and the outer nuclear layer. The outer photoreceptor segments are embeded in a pigmented epithelium, which serves to minimise light reflection within the eye. The phenotype of the albino rat is attributed to the absence of melanin in the pigmented epithelium rather than the iris (Lund *et al.*, 1974; Creel and Giolli, 1976).

A light stimulus reaching the layer of photoreceptor outer segments initiates a signal, which is then relayed to the bipolar and horizontal cells in the outer plexiform layer. Amacrine and bipolar cells project laterally to the inner plexiform layer and relate the signal to the ganglion cell layer. RGCs propagate the impulse via their axons in the optic nerve, to the primary visual centres in the thalamus and midbrain.

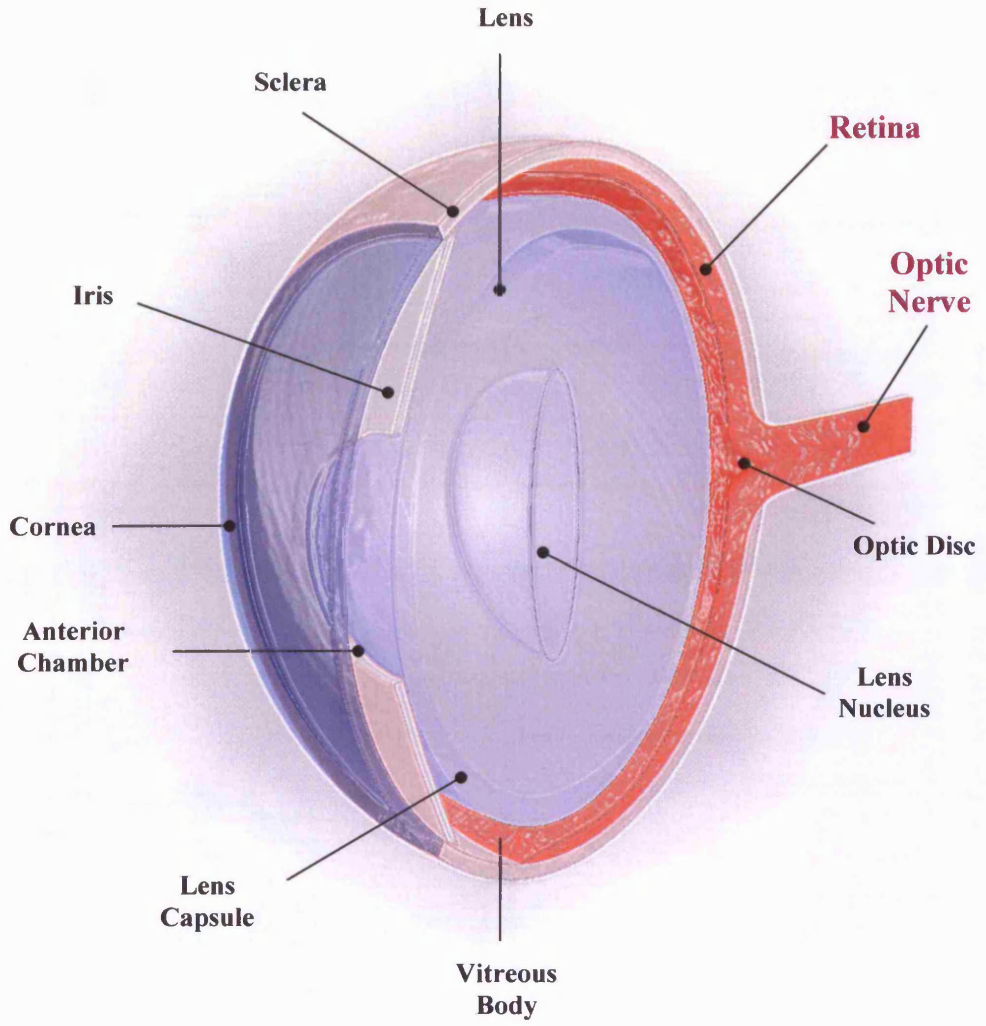


Figure 6.1.1-a: Anatomy of the rodent eye.

(Based on the dimentions given in Fischer *et al.* 2000). The rodent eye is surrounded by a tough fibrous outer layer, the sclera. Between sclera and retina lies the vascular choroid. The anterior chamber lies between the cornea and the lens and is filled with an aqueous humor of a slightly alkaline nature. The rodent lens is enclosed in a transparent and highly elastic capsule that occupies most of the posterior eye cavity and is suspended by ligaments attached to the cilliary body. The vitreous body, a viscous jelly-like fluid, separates the lens from the retina. The axonal processes of RGCs exit the retina via the optic disc and form the optic nerve. The optic nerves from each eye decussate at the optic chiasm and terminate in the superior colliculus and lateral geniculate nucleus.

Note: Intraocular structures are shown in proportion for an adult rat of approximately 200g.

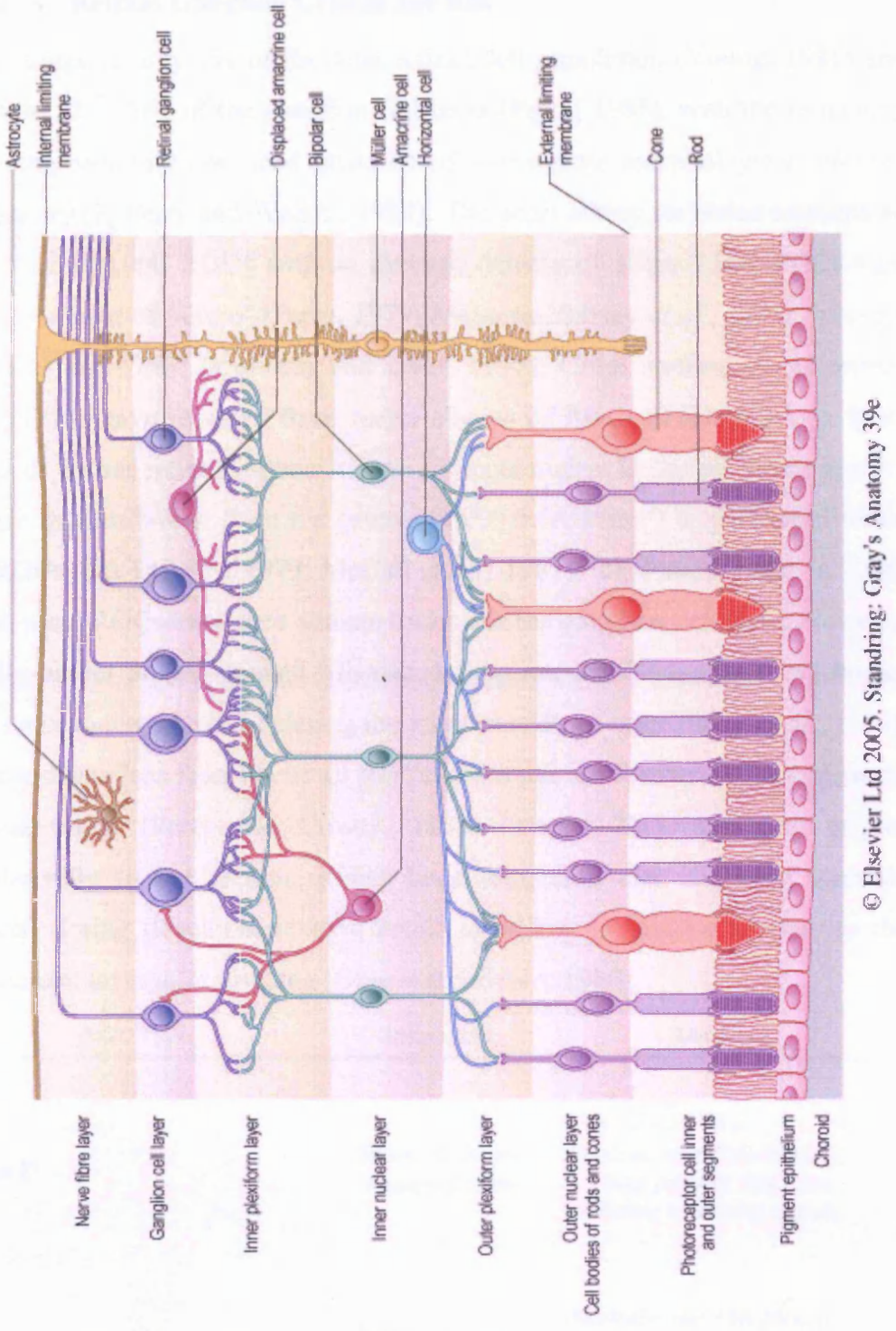


Figure 6.1.1-b: The laminar organisation in the retina.

Horizontal cells interconnect photoreceptors and bipolar cells and aid the function of amacrine cells. Bipolar cells co-ordinate the responses of many rods with a single RGC thus enhancing sensitivity (*summation*). Amacrine cells interconnect bipolar cells and RGCs. (Adapted from Grays's Anatomy, 2005, student licence agreement).

6.1.1.2 Retinal Ganglion Cells in the Rat

RGCs comprise only 1% of the total retinal cell population (Young, 1985) and constitute 57 – 70% of the ganglion cell layer (Perry, 1981), with the remaining cells being primarily displaced amacrine cells of various morphological subtypes (Hughes, 1977; Perry and Walker, 1980). The adult albino rat retina contains an average of 100,000 RGCs with an average density of about 2,000 RGCs/mm^2 over a total area of 50mm^2 (Perry, 1979; Mansour-Robaey *et al.*, 1994; Peinado-Ramon *et al.*, 1996; Isenmann and Bahr, 1997). Golgi studies on rat retinas (Perry, 1979) have revealed three major classes of RGCs (Table 6.1.1-a). RGC density in the rat retina displays a level of eccentricity. In the rat, there is a 5:1 decrease in distribution from the central (3,000 RGCs/mm^2) to peripheral retina (600 RGCs/mm^2) (Perry, 1979; McCall *et al.*, 1987). As demonstrated in Table 6.1.1-a, most RGCs have their somata located in the ganglion cell layer. However a small number of cells termed "displaced ganglion cells" have their cell bodies in the inner nuclear layer bordering the inner plexiform layer (Bunt *et al.*, 1974). They constitute less than 1% of all RGCs in the rat, are located in the peripheral temporal retina (Perry and Cowey, 1981; Perry, 1981), and they project controlaterally to the tectum. It has been suggested that they may become displaced during development as a result of failing to migrate in time to the ganglion cell layer after dividing (Drager and Olsen, 1980).

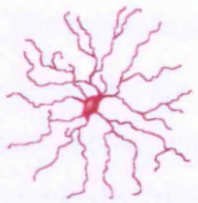
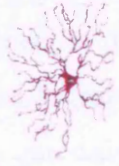
RGC Type		Dimentions	Dendrites
Type I		Soma: 16-32µm Axon Ø:0.9µm	2-4% of all RGCs Dendritic tree: 220-790µm. 3-7 long primary dendrites Radiating branching pattern
Type II		Soma: 11-22µm Axon Ø:0.6µm	Dendritic tree: 140-250µm 1-4 short primary dendrites Many short & spinny branches
Type III		Soma: 6-15µm Axon Ø:0.35µm	Dendritic tree: 410-540µm Fine, long primary dendrites Some branching present.

Table 6.1.1-a: Classes of retinal ganglion cells in the rat retina. Ø: diameter. Not to scale. Displaced ganglion cells are morphologiacally simillar to type I RGCs. (Perry, 1979)

6.1.1.3 Histological features of the intact rat optic nerve

It has been estimated that the optic nerve of an adult albino rat contains 100,000 axons (Fukuda *et al.*, 1982; Sefton and Lam, 1984). RGC axons course along the optic fibre layer on the top of the ganglion cell layer and exit the retina at the optic disc. Until they reach the optic disc, axons remain unmyelinated (transparent) and the nerve bundles are surrounded only by astrocytes. However, in the orbital optic nerve virtually all axons become myelinated by oligodendrocytes (Fukuda *et al.*, 1982). Electron micrographs have demonstrated that axons are tightly packed within the optic nerve such that no connective tissue and little extracellular space is present (Dezawa and Adachi-Usami, 2000). The optic nerve contains the same glial organisation as the rest of the CNS with oligodendrocytes, astrocytes and microglia, organised in a manner similar to all other CNS white matter tracts. Their cell bodies lie in rows, parallel with the fascicles of RGC axons (Bussow, 1980; Suzuki and Raisman, 1992). Optic nerve astrocytes are interconnected by gap junctions with their longitudinal and radial processes forming an integrated meshwork (Dr G. Campbell, unpublished observations). They have numerous processes, some of which end as footplates just beneath the pial surface, forming the glial limiting membrane. There astrocytes appear thicker and have basement membranes (Massa and Mugnaini, 1982; Dezawa and Adachi-Usami, 2000).

6.1.1.4 The retinofugal pathway in the rat

RGCs represent the only output from the retina relaying visual stimuli to the primary visual centres via the retinofugal pathway. All RGC axons exit each retina at the optic disc where they cluster together forming the optic nerve. Both optic nerves meet at the optic chiasm where RGC axons decussate and form the optic tract. The degree of decussation at the optic chiasm varies between species. In the albino rat only 1% of all RGCs project to ipsilateral retino-recipient regions involved in the binocular representation of the visual field (Lund, 1975; Dreher *et al.*, 1985). This small percentage of RGCs originate from the temporal retina (Isenmann *et al.*, 2003). In albino rats, information from the ipsilateral eye is relayed via commisural projections from the controlateral visual cortex (Silveira *et al.*, 1989). After the optic chiasm, optic tract axons peel off to form

connections with a number of hypothalamic and pretectal nuclei before finally terminating in the midbrain tectum. Hypothalamic targets include the suprachiasmatic nucleus (SCN), which lies just dorsally of the optic chiasm. Receiving direct retinal innervation, the SCN is thought to be responsible for synchronising circadian rhythms with the daily light-dark cycle. Pretectal nuclei such as the olivary pretectal nucleus (OPT) participate in both direct and consensual pupillary light reflexes by receiving information relevent to the light intensity reaching the retinal surface (Tracey, 1994). In addition, approximately 30% of all rat RGCs send collaterals to the lateral geniculate nucleus (LGN) in the dorsal thalamus (Dreher *et al.*, 1985). By receiving direct synaptic input from the retina and relaying information to the visual cortex, LGN is the gateway to conscious visual perception. Unlike primates, the LGN of rodents does not demonstrate the same level of structural complexity. In almost all primates, controlateral/ipsilateral and nasal/temporal RGC projections synapse with LGN neurons located in six separate layers (Hubel and Livingstone, 1990; Bear *et al.*, 2001). In the rat however, only a very basic level of segregation exists keeping controlateral and ipsilateral synapses in just two separate groups of LGN neurons.

The final synaptic target of optic nerve fibres is the superior colliculus located in the midbrain tectum (Perry *et al.*, 1983). RGC axons synapse in the superficial layer of superior colliculus (stratum griseum superficiale). Receiving a direct input from RGCs the superior colliculus, via the cranial nerves, coordinates the eye muscles for saccadic and smooth pursuit movements. During development, axonal guidance molecules such as ephrins and their receptors follow graded expression patterns in both retina and superior colliculus (Sperry, 1963; Knoll *et al.*, 2001; Isenmann *et al.*, 2003; Oster and Sretavan, 2003). As a result, retinotectal projections are highly organised topographically (Mueller, 1999) and are conserved between species (Thanos and Mey, 2001; Heiduschka *et al.*, 2001). A retinotectal map with both a dorsal-ventral and nasal-temporal orientation is used to describe this retinotopic relationship. For example, ventral retina axons project to dorsal superior colliculus while dorsal retina axons project to ventral superior colliculus. Similarly, nasal retina projects to posterior while temporal retina projects to anterior superior colliculus (Isenmann *et al.*, 2003).

6.1.2 Responses of the optic nerve to axotomy

Several types of axonal injury can lead to RGC degeneration. These have been classified as: optic nerve compression (or crush), transection, glaucomatus optic neuropathy, papilledema, inflammation and de-myelination (Farkas and Grosskreutz, 2001; Levin and Gordon, 2002). Even though glaucomatus optic neuropathy is the most clinically relevant axonal injury, optic nerve transection and compression are by far the most extensively characterised experimental degeneration and regeneration models in the rodent visual system. The following sections outline the morphological and molecular changes that adult rat RGCs undergo following optic nerve injury, with emphasis on the compression model.

6.1.2.1 Effects of optic nerve compression on adult rat RGCs

Optic nerve injury results in RGC loss, the extent of which varies depending on the type and severity of the lesion, proximity to the retina and the age of the animal. Well-defined crush lesions divide the optic nerve into the distal segment, between the lesion and the optic chiasm and the proximal segment between the lesion and the retina. RGC responses following axotomy share a degree of similarity between transection (Villegas-Perez *et al.*, 1993) and compression (Berry *et al.*, 1999; Campbell *et al.*, 1999; Selles-Navarro *et al.*, 2001) injury models. RGC death appears to follow a well-defined temporal pattern regardless of the way the lesion was inflicted. Complete transection of the optic nerve results in extensive ischemia and necrosis accompanied by a large region of cavitation extending proximally to the lesion site (Grafstein and Ingoglia, 1982). Although a crush lesion has a smaller injurious impact on morphology and RGC viability, scar formation and cavitation still develops in the central optic nerve extending proximally (Berkelaar *et al.*, 1994) to the optic disc.

24 hours following a crush lesion, the majority of RGC axons appear to have variably retracted 30 to 200μm from the lesion site while very little evidence of an injury can otherwise be seen (Frank and Wolburg, 1996). One possible explanation for this axonal retraction stems from the following studies, performed

on white matter axonal injury. These suggest that voltage-gated sodium channels might provide a route for sodium influx into axons that triggers reverse operation of the Na^+/Ca^{2+} exchanger (NCX) and subsequent influx of damaging levels of intra-axonal Ca^{2+} (Craner *et al.*, 2004), ultimately leading to cytoskeleton depolymerisation (Meller, 1987; LoPachin and Lehning, 1997; Campbell *et al.*, 1999). Overall, there is no significant decline in retinal cell numbers (Misantone *et al.*, 1984) at this time.

At 7 days post lesion, neurofilament immunoreactivity (NF-IR) begins to decline in the distal stump. This decline is gradual, signalling the onset of Wallerian degeneration (Ohlsson *et al.*, 2004). Towards the end of the first week, RGC loss increases with 68% (Selles-Navarro *et al.*, 2001) of crushed RGCs surviving. Axons proximal to the lesion begin to sprout vigorously demonstrating increased GAP43 immuno-reactivity (Ohlsson *et al.*, 2004). These sprouts appear very small with a diameter ranging between 100 and 200nm (Campbell *et al.*, 1999). Unexpectedly, the Ca^{2+} influx, reported to be responsible for the axonal retraction observed at 24 hours post lesion, is also thought to contribute to this axonal sprouting. There is evidence to suggest that in fact this elevation in intracellular Ca^{2+} concentrations activate a group of Ca^{2+}-depended proteases known as calpains. Even though their role is controversial (Wang and Yuen, 1994), several studies have suggested that calpains play a role in membrane resealing after injury (Xie and Barrett, 1991) and in restoration of dendritic structures after excitotoxic injury (Faddis *et al.*, 1997). In addition, transient and localised elevation in cytoplasmic proteolytic activity is capable of inducing growth cone formation (Ziv and Spira, 1997; Ziv and Spira, 1998). By 14 days post crush, only 28% of RGCs are still viable (Selles-Navarro *et al.*, 2001). In the case of a complete optic nerve transection, the number of surviving RGCs falls to 10% at two weeks post injury (Bray *et al.*, 1991; Villegas-Perez *et al.*, 1993; Berkelaar *et al.*, 1994; Zeng *et al.*, 1994; Selles-Navarro *et al.*, 2001). This vigorous sprouting noted by the end of 7 days post injury, becomes largely abortive once the axons encounter the scar tissue of the lesion. Very few spontaneously regenerating axons can be seen entering the lesion and extending distally up to 200μm while one or two axons were found to have extended up to 700μm distally (Campbell *et*

al., 1999). The majority of regenerating axons appear to run parallel to the longitudinal axis of the optic nerve. Some of them however appear to run indirectly, making sharp turns, or occasionally looping back on themselves. At 20 days post crush the distal segment of the optic nerve appears devoid of GAP43 immunoreactivity, while abundant neurofilament (RT-97) positive debris can be seen (Berry *et al.*, 1996). The few spontaneously regenerating axons found at 200-900μm distal to the lesion, are unmyelinated (Campbell *et al.*, 1999). Eight weeks after crush injury, only low levels of neurofilament immunoreactivty are detected in the distal stump (Ohlsson *et al.*, 2004). Regenerating axons extending between 1.5 to 2.0mm distally, were found in clusters of 2-10 surrounded by astrocytic processes beneath the glia limitans.

RGCs continued to demonstrate a very limited ability for long term regeneration with one or two axons found at 6mm and 8mm distally at four and sixteen months post lesion respectively (Campbell *et al.*, 1999). Approximately 5% of RGCs still survive 20 months after intraorbital nerve section (Villegas-Perez *et al.*, 1993).

6.1.2.2 The glial reaction to optic nerve injury

Similarly to other tracts of the CNS, the permanent impairment that follows a crush injury involves alterations in non-neuronal cells including the formation of a glial scar. The cellular events leading to the formation of the glial scar appear to occur within defined chronological boundaries. Even though there appear to be differences in the literature regarding the onset and extent of morphological alterations within the crushed optic nerve, these are primarily due to the different experimental approaches used to inflicting the crush lesion. A crush lesion inflicted with a suture ligature (microcrush) (Selles-Navarro *et al.*, 2001) produces a far more focal point of lesion compared to the more extensive disruption generated when applying constant pressure with a fine pair of forceps (Ohlsson *et al.*, 2004). However, qualitatively the cellular alterations that follow are very similar.

Within 24 hours of crushing using fine forceps, the optic nerve appears swollen with an intact pia matter. The cellular architecture of the nerve is disrupted including the destruction of blood vessels. The pattern of GFAP immunoreactivity denotes the absence of astrocytes from the lesion centre and their presence in the immediate proximal and distal zones. No microglia or macrophages were found to be present in either the lesion centre or in the proximal or distal segments (Frank and Wolburg, 1996) after staining for either ED1 or lysozyme. ED1-positive/lysozyme-positive cells of round shape enter the lesion centre after 2 days with a higher concentration found around disrupted blood vessels. Immunocytochemistry revealed that the ED1 antigen was localised to the membranes of phagosomes, consistent with the role of these cells in clearing debris. In the case of a microcrush a clearly defined focal lesion is produced that appears to be populated by leptomeningeal cells (Selles-Navarro *et al.*, 2001). These authors propose that the presence of leptomeningeal cells, migrating into the lesion site from the pia matter, points to their potential implication in creating a non-permissive environment. Indeed, leptomeningeal cells have previously been shown to be non-permissive to axonal growth (Hirsch and Bahr, 1999). In addition, NGF, secreted by the large number of astrocytes that re-populate the lesion site (Ishikawa *et al.*, 1991) a week post injury, has been shown to stimulate process outgrowth in these cells (Frisen *et al.*, 1998).

Five to seven days after the lesion microglia, macrophages, astrocytes and fibroblasts begin to infiltrate the crush site. Co-localisation of BrdU and OX42 immunoreactivity has confirmed intense proliferation of microglia (Ohlsson *et al.*, 2004). The numbers of ED1-positive/lysozyme-positive cells peak and increase in diameter due to the incorporation of large amounts of cellular debris. ED1-positive/lysozyme-negative activated microglia and macrophages with a branched morphology begin to populate the distal segment of the crushed optic nerve but demonstrate little phagocytic activity. Astrocytes begin to re-enter the lesion site while at the edges of the lesion, reactive astrocytes enclose axonal and myelin debris (Frank and Wolburg, 1996).

Two weeks after injury, blood vessels extensively invade the lesion site, as indicated by an increase in laminin immunoreactivity and branch both distally and proximally from the centre of the lesion (Selles-Navarro *et al.*, 2001). Astroglia undergo hypertrophy and proliferate based on BrdU and GFAP co-localisation studies (Ohlsson *et al.*, 2004). Over the next 20 to 30 days, the distal segment is packed from the lesion edge with myelin basic protein (MBP, a general myelin marker) and myelin proteolipid protein (PLP or lipophilin, a CNS specific myelin marker) containing degenerating myelin. The actual lesion site contains islands of degenerating myelin. Amongst the degenerating myelin, CAII-positive oligodendrocytes are present in the distal and proximal segments but not at the lesion site. The basal lamina, which appears to be continuous with the glia limitans externa, is underlaid by several layers of astrocyte processes and a single layer of end-feet (Berry *et al.*, 1999). Reactive astrocytes are present in both segments of the optic nerve. Few astrocytic processes bridge the lesion, which contains unstained regions circumscribed by GFAP positive glia limitans. ED1-positive macrophages accumulate in the lesion and perilesion regions of the proximal and distal segments. However, ED1-positive microglia are found in the rest of the optic nerve (Berry *et al.*, 1996).

After 8 weeks, a meshwork of blood vessels is in place, creating a mechanical barrier to regenerating axons. GFAP immunoreactivity is restored within the lesion site and appears uniform (Selles-Navarro *et al.*, 2001). Microlia and astrocytes still appear to proliferate along the entire portion of the distal optic nerve (Ohlsson *et al.*, 2004). The glial scar that has formed is encapsulated by a convoluted basal lamina whose well vascularised central core of connective tissue (Fawcett and Asher, 1999) is rich in heamatogenous macrophages, astrocytes, reactive microglia and fibroblasts. The precise sequence of events following optic nerve trauma can vary and largely depends on the site and severity of the lesion (Fawcett and Asher, 1999). Nevertheless, the glial scar that forms in the optic nerve presents a significant barrier to RGC axonal regeneration. This is clearly evident when considering the fact that not even olfactory bulb axons, which have an exceptional capacity for regeneration, can regenerate their axons through a transplanted optic nerve glial scar (Anders and Hurlock, 1996).

6.1.3 Reasons for the regenerative failure of adult RGCs

After axotomy RGCs demonstrate a capacity for spontaneus regeneration, albeit fruitless. This has been reported *in vivo* after optic nerve crush and optic nerve transection (Zeng *et al.*, 1994; Campbell *et al.*, 1999). Also, retinal explants from adult rats that have been pre-lesioned by a crush injury (Tsai *et al.*, 1998) or transection (Johnson *et al.*, 1988a; Johnson *et al.*, 1989), do exhibit an enhanced intrinsic ability to extend neurites compared to non-pre-lesioned animals (Johnson *et al.*, 1988b). However, this ability declines rapidly 10 days post axotomy (Tsai *et al.*, 1998). The factors that render fruitless any spontaneous RGC regenerative attemps are, as in the rest of the CNS, multifactorial. Retrograde transport of axotomy-induced signals and loss of axonal target derived trophic support are thought to contribute to the initiation of intrinsic signalling pathways, ultimately leading to RGC apoptosis (Berkelaar *et al.*, 1994; Garcia-Valenzuela *et al.*, 1994). In addition, myelin-associated and other glial scar inhibitory molecules are additional factors detrimental to regenerative attempts.

6.1.3.1 Loss of target-derived neurotrophic support

RGCs depend on the retrograde trophic support they receive from their targets in order to survive (Purves *et al.*, 1988), inkeeping with other CNS neurons. This is supported by the relationship exhibited between the length of the surviving optic nerve stump and the speed of cell death onset (Villegas-Perez *et al.*, 1993). In addition, trophic molecules present in an axotomised optic nerve can still exert their neuroprotective effect on the cell bodies of axotomised RGCs, as suggested by the fact that a pre-lesioned transplanted optic nerve in the vitreous is capable of transiently delaying the onset of RGC death in adult hamsters (Cho *et al.*, 2001). The longer the surviving optic nerve, the more neurotrophic factors are present to support survival (Herdegen *et al.*, 1997). The mechanism underlining this loss of trophic support is poorly understood. There is growing evidence to imply that loss of target-derived trophism is not the sole factor playing a part in the resulting cell death. Firstly, kainate lesion of the superior colliculus, with sparing of RGC axons, does not lead to RGC death (Carpenter *et al.*, 1986) even

though sparing of connections to other retinorecipient targets cannot be ruled out in this study. Secondly, intraoccular administration of FGF (Cui *et al.*, 1999), CNTF, BDNF (Mey and Thanos, 1993; Mansour-Robaey *et al.*, 1994; Di Polo *et al.*, 1998) or GDNF (Klocker *et al.*, 1997) can support survival of axotomised RGCs but only transiently.

The inability of neurotrophic factors to sustain a functional effect could be attributed to the failure of injured RGCs to respond to these signals. This could be explained by the existence of different Trk isoforms that although capable of binding their high affinity ligands are not capable of transducing the stimulatory signal. The levels of TrkA and TrkB mRNA begins to gradually decline folowing optic nerve injury, reaching a minimum after 1 month (Cui *et al.*, 2002; Cheng *et al.*, 2002). This observation, could explain the ephemeral effect of NT4/5 and BDNF administration in enhancing the survival of injured RGCs (Clarke *et al.*, 1998; Di Polo *et al.*, 1998). Similarly, despite the fact that TrkC levels in injured RGCs appear to remain unaltered for up to five weeks post lesion (Cui *et al.*, 2002), administration of NT3 has a minimal impact on both RGC survival and axonal regeneration (Peinado-Ramon *et al.*, 1996; Sawai *et al.*, 1996). Several TrkC forms have been identified including one that completely lacks a catalytic domain (Tsoulfas *et al.*, 1993). It is therefore logical to hypothesize that failure of RGCs to regenerate involves not only the declining access to factor availability but also to a reduced ability to respond to any potential trophic signals. This reduced responsiveness of axotomised RGCs to trophic signals has also been attributed to declining cAMP levels (Shen *et al.*, 1999), caused in turn by the loss of synaptic input (Harwerth *et al.*, 2002). It would therefore seem possible that the elevation of cAMP levels on its own would be enough to enhance survival and regeneration of injured RGCs, as it would indirectly lead to a higher responsiveness to trophic factors. However, there are conflicting reports on the ability of cAMP administration in promoting survival and/or regeneration of injured RGCs *in vivo*. On the one hand, cAMP elevation alone, by means of the CPT-cAMP (8-(4-Chlorophenylthio)adenosine-3',5'-cyclic monophosphate-cAMP or CPT-cAMP) analogue administered intraocularly, was reported not to be sufficient to increase either the number of surviving RGCs or the number of

regenerating axons into a peripheral graft (Cui *et al.*, 2003a). When cAMP elevation is accompanied by CNTF administration however, a significant increase in both survival and axonal regrowth is observed. This finding is in agreement with previous reports where adult RGCs in culture were found to be intrinsically unresponsive to trophic factor stimulation unless they were depolarised (Meyer-Franke *et al.*, 1998), or levels of their intracellular cAMP were elevated pharmacologically. *In vitro* studies are suggestive of a possible involvement of cAMP in increasing the recruitment of TrkB receptors (Meyer-Franke *et al.*, 1998), hence indirectly enhancing the responsiveness of axotomised RGCs to the available neurotrophic agents. Indeed, when TrkB gene transfer was combined with BDNF delivery, RGC survival was increased by 76% three weeks after axotomy as opposed to 10% of RGCs surviving without treatment (Cheng *et al.*, 2002). *In vitro* studies on rat cerebellar granule neurons have demonstrated that elevation of intracellular cAMP levels leads to the phosphorylation and inhibition of glycogen synthase kinase 3β (GSK-3β) by protein kinase A (PKA) (Li *et al.*, 2000). These findings, hint to the fact that RGC death following axotomy can not be explained solely by the loss of retrograde, glial and target derived trophic stimuli but also by a decline in trophic responsiveness (Shen *et al.*, 1999). On the other hand, recent reports contradict the above findings. Monsul and coleagues report that a single intraocular injection of the cAMP analogue Dibutyryl-cAMP (N^6, 2'-O- Dibutyryladenosine-3', 5'-cyclic monophosphate-cAMP or Db-cAMP) is sufficient to promote RGC axon regeneration in the adult rat crush model but had no effect in preventing RGC death (Monsul *et al.*, 2004).

However, several reports point out the pitfalls regarding the use of both Db-cAMP and CPT-cAMP. Firstly, in its native form Db-cAMP is not a substrate for PKA and needs to split off one of its butyryl groups in order to adopt the kinase active form of monobutyryl-cAMP. The released butyrate can carry out its own distinct effects in second messenger pathways and can shift the focus from PKA (Rivero and Adunyah, 1998). Butyrate can alter gene expression by suppressing histone deacetylation leading to increased gene expression. This effect of butyrate appears to be type specific and in cerebellar granule neurons for example, sodium butyrate leads to apoptosis by the activation of caspase-3 protease (Salminen *et*

al., 1998). This is indicative of the fact that a different pathway may be at play inducing the effects of sodium butyrate considering that CPT-cAMP can promote survival of cerebellar granule neurons by directly activating PKA (Li *et al.*, 2000). Since the effects of Db-cAMP can be attributed to the butyryl moiety of this compound rather than to the cyclic nucleotide itself, it would be more appropriate that the use of Db-cAMP be accompanied by control experiments using sodium butyrate. This was not performed in the Monsul study and therefore doubt can be cast over the correlation of the observed regeneration and the direct involvement of PKA activation. Secondly, the 8-CPT-cAMP analogue is also associated with pitfalls as it is reportedly not specific for cAMP. It has been implicated in the activation of cGMP-dependent protein kinase (Geiger *et al.*, 1992) and it raises basal cGMP levels by inhibiting phosphodiesterase 5 (Connolly *et al.*, 1992). A more specific analogue of cAMP such as the Sp-isomer of 8-CPT-cAMP (Sp-5,6-DCl-cBIMPS) could provide a more definitive answer on the involvement of cAMP levels in promoting survival and/or regeneration of axotomised RGCs.

6.1.3.2 Death signals in axotomised RGCs

The retrograde RGC death that follows optic nerve injury, appears to display the characteristic trademarks of apoptosis (Berkelaar *et al.*, 1994; Garcia-Valenzuela *et al.*, 1994; Isenmann *et al.*, 1997), including cell body shrinkage and DNA fragmentation in the absence of an inflammatory response (Gavrieli *et al.*, 1992; Garcia-Valenzuela *et al.*, 1994). However, it is worth noting that some necrosis also takes place as a result of the immune response taking place at the site of a mechanical lesion. Axotomy also initiates a cascade of microglia-mediated responses, which culminate in degradation of injured, but obviously surviving neurons (Thanos *et al.*, 1993).

Initiation of the apoptotic cascade can occur either via the intrinsic mitochondrial pathway (Kroemer *et al.*, 1997) or by the activation of cell surface death receptons (Ashkenazi and Dixit, 1998). Both routes exert their effects via two main gene families: the caspases (Kermer *et al.*, 1999; Kermer *et al.*, 2000) and

the Bcl-2 family (Kroemer, 1997) (Figure 6.1.3-a). In response to an apoptotic signal, an alteration in the permeability of the membranes of the cell's mitochondria occurs. This event causes the translocation of the apoptogenic protein cytochrome c into the cytoplasm. This in turn activates death-driving proteolytic proteins known as caspases. The role of caspaces in initiating secondary RGC death has been well documented. Caspase-3 (CASP3) and it's activator CASP9, have been shown to be activated in degenerating RGCs (Kermer *et al.*, 1999; Kermer *et al.*, 2000). Interestingly, the timing of their activation, pin pointed at 3 days post axotomy, coinsides with the onset of RGC death. It is thought that the PI3K/Akt pathway may serve as an endogenous regulator of caspase activation in axotomized RGCs (Cheung *et al.*, 2004). The use of CASP3 inhibitors can reduce the extent of RGC death but the effect is short-lived (Kermer *et al.*, 1999), further indicating the complexity of the mechanisms underlying neuronal death and survival following trauma.

The Bcl-2 family of proteins, whose members may be pro-apoptotic (BAX, BAD, BID, BAK, BCL-X_S, BIK, BIM, HRK) or anti-apoptotic (BCL-2, BCL-X_L, BCL-W, MCL-1, A1), regulate cell death by controlling mitochondrial membrane permeability during apoptosis (Farrow and Brown, 1996; Kroemer, 1997). Overexpression of the apoptosis repressor Bcl-2 in transgenic mice, supports sustained survival of RGCs after intracranial optic nerve transection (Cenni *et al.*, 1996). However, studies in Bcl-2 knockout mice (-/-), demonstrated that it is not a requirement for the survival of the subpopulation of axotomised adult RGCs that manage to survive unaided 14 days after axotomy (Dietz *et al.*, 2001). These results further support the notion that the naturally low levels of Bcl-2 do not play a detrimental role in delaying the onset of RGC degeneration. Similarly, over expressing the anti-apoptotic protein Bcl-XL by means of an adenoviral vector, may promote axonal regeneration on axotomised RGCs, but fails to ensure functional regeneration (Kretz *et al.*, 2004).

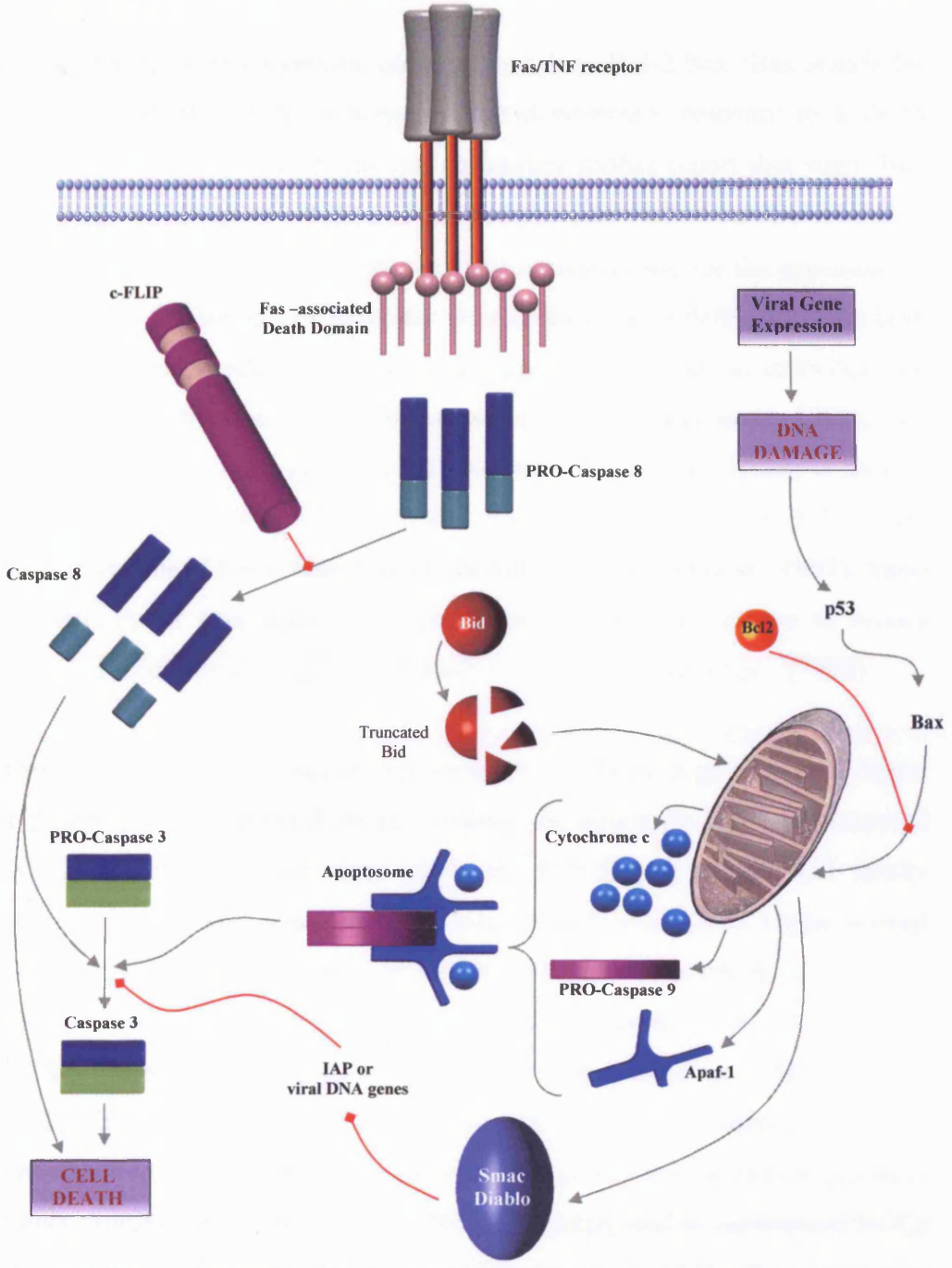


Figure 6.1.3-a: Role of mitochondria in apoptotic signalling.

There are two major apoptotic pathways in mammalian cells: The death receptor mediated pathway via the activation of caspase 8 and the mitochondria pathway via the activation of caspase 9. Cytochrome c is released from mitochondria in response to apoptotic signals and activates the Apoptosis activating factor-1 (Apaf-1), a protease also released from mitochondria. Activated Apaf-1 activates caspase-9 and the rest of the caspase pathway. Smac/DIABLO is released from mitochondria and inhibits Inhibitors of Apoptosis Proteins (IAP) that normally interact with caspase-9 to inhibit apoptosis (Srinivasula *et al.*, 2001). The TNF family of receptors cause apoptosis by directly activating the caspase cascade, but can also activate Bid, a Bcl-2 family member. This in turn initiates mitochondria-mediated apoptosis. Bax, another Bcl-2 family member, is activated by this pathway and localises to the mitochondrial membrane. Bcl-2 prevent membrane pore formation thus blocking apoptosis. AIF (Apoptosis inducing factor) is another mitochondrial factor that is released into the cytoplasm to induce apoptosis in a non caspase dependent manner (Joza *et al.*, 2001). (Diagram adapted from http://www.biocarta.com).

The ratio of anti- to pro-apoptotic molecules such as Bcl-2/Bax (Bax stands for Bcl-2 associated protein X), determines the downstream response to a death signal (Oltvai *et al.*, 1993). Oltvai and colleagues further report that when Bax predominates, apoptosis is accelerated and the protective effect conferred by Bcl-2 is counteracted. Bax induction, shown to be a prerequisite for the execution of RGC apoptosis following ON axotomy (Isenmann *et al.*, 1999b), involves both Bcl-2 and p53 and results in a downstream process that leads to mitochondrial dysfunction as well as activation of caspases (Miyashita and Reed, 1995). Bax protein, expressed in most ganglion cells at moderate baseline levels, is sharply increased as early as 30 min after a crush, reaches peak levels after 3 days, and remains up-regulated for at least 1 week thereafter (Isenmann *et al.*, 1997). Intravitreal delivery of Bax antisense oligonucleotides has been shown to reduce axotomy-induced retinal ganglion cell death *in vivo* (Isenmann *et al.*, 1999b).

A variety of transcription factors, belonging to the basic region leucine zipper super family (bZip), are involved in initiating the apoptotic fate of axotomised RGCs. These include, among many others, the JUN family and the ATF family (Karin *et al.*, 1997; Hai *et al.*, 1999; Shaulian and Karin, 2002). Under normal conditions, c-Jun is not expressed in RGCs. Prolonged expression of c-Jun has been linked to apoptosis in many models of neurodegeneration both *in vitro* and *in vivo*. For example, overexpression of c-Jun results in neuronal apoptosis (Ham *et al.*, 1995) but only *in vitro* (Lu *et al.*, 2003) while suppression of c-Jun prevents death in NGF deprived sympathetic embryonic neurons *in vitro* (Estus *et al.*, 1994). Additionally, c-Jun and GAP43 are co-expressed in axotomised RGCs found regenerating into a peripheral graft (Schaden *et al.*, 1994), thus supporting later reports that c-Jun is involved in regulating the expression of GAP43 (Lu *et al.*, 2003). However, the extent and contribution of c-Jun in the degenerative and regenerative process of axotomised RGCs is not clear as it appears to modulate both pro-apoptotic and regeneration signals (Herdegen *et al.*, 1997). Axotomy causes a rapid induction of c-Jun which persists in those RGCs that manage to survive and/or regenerate (Robinson, 1994; Hull and Bahr, 1994a), as well as RGCs that appear to undergo apoptosis (Isenmann and Bahr, 1997). Survival and expression of c-Jun in rat RGCs appears to be independently regulated by the

length of the optic nerve stump, with c-Jun up-regulation beginning later and declining faster after distal axotomy compared to proximal transection of the optic nerve (Hull and Bahr, 1994a). c-Jun expression begins to decline at 8 days post axotomy but can be re-induced if regenerating RGC axons are subjected to secondary axotomy (Hull and Bahr, 1994b). Moreover, when retinas from *c-jun* deficient mice were co-cultured with the superior colliculus of newborn rats, they managed to survive and elongate their axons to the same extent as wild type retinas (Herzog *et al.*, 1999), indicating that c-Jun is essential neither for programmed cell death nor axonal elongation. This dual role of c-Jun can be explained if the observed upregulation in both degenerating and regenerating RGCs is interpreted as a common cell body response, with the eventual fate of axotomised RGCs being decided by the specific homo/hetero-dimers formed by c-Jun, and the phosphorylation of these complexes by Jun N-terminal kinases (JNK), which are part of the cellular stress response (Herdegen *et al.*, 1997). There is evidence to support the notion that N-terminal phosphorylation of c-Jun contributes to RGC apoptosis following axotomy, since blocking both the c-Jun serine phosphorylation sites affords a larger number of surviving RGCs (Yoshida *et al.*, 2002). Also, it is reported that c-Jun can form heterodimers with the ATF family of bZip transcription factors and, via binding to specific DNA sequences, control the fate of axotomised RGCs. ATF-3 for instance has emerged as a modulator of c-Jun transactivation showing a high specificity for a subset of surviving RGCs following an optic nerve crush (Takeda *et al.*, 2000).

6.1.3.3 Intrinsic growth capacity of axotomised RGCs

In addition to the factors outlined above, like all other neurons of the CNS, RGCs also demonstrate the same low levels of intrinsic growth capacity. Decreased anterograde transport of cytoskeletal components to the site of injury hinders any potential for axonal regeneration. The level of tubulin and neurofilament protein in non-regenerating axotomised RGCs is reportedly significantly lower than that found in regenerating RGCs, following of a peripheral graft (Fournier and McKerracher, 1995). Notably, slow component a (Sca) transport of tubulin in a crushed optic nerve decreases from a normal rate of 0.5 mm/day to 0.06 mm/day,

resulting in a failure of tubulin and neurofilament levels to reach the site of axonal injury (McKerracher *et al.*, 1990b). In RGCs that do regenerate into a peripheral nerve graft anastomosed to the transected optic nerve, the rate of Sca increases to 1 mm/day (McKerracher *et al.*, 1990a).

Another molecule implicated in the decreased growth capacity of RGCs is GAP43, thought to influence the re-organisation of the axonal cytoskeleton (Benowitz and Routtenberg, 1997) in a mechanism involving its phosphorylation by protein kinase C (PKC) (Akers and Routtenberg, 1985). GAP43 is not normally present in adult RGCs but a small proportion appears to express the protein following axotomy (So and Yip, 1998). It is these GAP43 expressing RGCs that appear capable of regenerating their axons into a peripheral nerve graft anastomosed to the transected optic nerve, demonstrating a direct link between GAP43 expression and axonal regeneration (Schaden *et al.*, 1994; Ng *et al.*, 1995). Studies in GAP43 (-/-) null mice reveals that even though RGC axonal elongation pauses for 6 days at the optic chiasm, axons still reach their retinorecipient targets (Strittmatter *et al.*, 1995), thus demonstrating that GAP43 is not essential for axonal elongation but plays a role in axon guidance and path finding, at least during development (Zhang *et al.*, 2000). This observation is in accordance with previous data, which suggest that over-expressing GAP43 in the adult CNS is not enough to support regeneration (Mason *et al.*, 2000). Studies using GAP43 promoter elements in transgenic zebrafish, indicate that the signaling pathways regulating GAP43 activation after CNS axotomy are different to those acting during developmental axonal elongation (Udvadia *et al.*, 2001). In mammals, overexpression of GAP43 in the rat cerebellum using the Purkinje specific L7 promoter, enhances sprouting of Purkinje axons after knife cut injury, but does not promote axonal regeneration into growth permissive substrates of embryonic neurons grafted within the lesion site (Buffo *et al.*, 1997). Hence, gene expression during axonal regeneration may involve a separate pathway that may share a degree of similarity with that acting during development (Bulsara *et al.*, 2002; Isenmann *et al.*, 2003). Interestingly, no RGC axotomy experiments have been performed using GAP43 transgenic animals. It is also worth noting that sciatic nerve injury in GAP43 overexpressing transgenic mice, results in

motorneuron death, but has no effect in non-transgenic animals. The authors suggest that continued growth of motor axons renders adult motorneurons susceptible to nerve injury and compromises their long-term survival potential (Harding *et al.*, 1999). Whether this is true for RGCs or not has not been addressed, but it is a factor that should be considered in regenerative attempts such as long-term delivery of GAP43 as a transgene.

6.1.3.4 Growth hindering molecules in the glial scar

In addition to the loss of trophic support and reduced growth capacity, surviving RGCs are also faced with a plethora of inhibitory molecules present in astrocytes (McKeon *et al.*, 1991; Bahr *et al.*, 1995) myelin and oligodendrocytes (Berry, 1982; Caroni *et al.*, 1988; Schwab and Caroni, 1988; Caroni and Schwab, 1988b; Bandtlow *et al.*, 1990; Bahr and Przyrembel, 1995), anyone of which appears sufficient to inhibit regeneration (Wang *et al.*, 2002a). These include MAG (McKerracher *et al.*, 1994; Mukhopadhyay *et al.*, 1994; Li *et al.*, 1996), OMgp (Mikol *et al.*, 1988; Wang *et al.*, 2002b; Vourc'h and Andres, 2004) as well as the Nogo family of proteins (Chen *et al.*, 2000; GrandPre *et al.*, 2000), acting via their high affinity receptor Nogo 66 (Fournier *et al.*, 2001) and $p75^{NTR}$ (Domeniconi *et al.*, 2002; Wang *et al.*, 2002a), all of which have been discussed in Chapter 1. Here, the focus is directed to evidence of the involvement of these molecules in inhibiting regeneration of axotomised RGCs.

When axonal growth cones from mature rat RGCs are confronted by rat central myelin *in vitro*, they either undergo growth cone collapse or avoid the myelin by altering the direction of axonal elongation (Vanselow *et al.*, 1990; Bahr and Przyrembel, 1995). Hence, just like for the rest of the adult CNS neurons, myelin does present an overall non-permissive environment to regeneration for axotomised RGC, at least *in vitro*. Neurons co-express Nogo-A, NgR and OMgp (Habib *et al.*, 1998) while MAG is primarily localised in glia (Martini, 1994). In their uninjured state, RGCs strongly express Nogo-A, found primarily in the somata rather than axons either in the fibre layer of retina or in the optic nerve (Huber *et al.*, 2002; Hunt *et al.*, 2003), possibly due to the rapid trafficking of

Nogo-A along the axon (Hunt *et al.*, 2002a). Nogo66 is present in the ganglion cell layer of the retina and in scattered cells within the optic nerve, presumed to be oligodendrocytes (Chen *et al.*, 2000; Huber *et al.*, 2002; Wang *et al.*, 2002b; Hunt *et al.*, 2002b). Four days after an optic nerve crush, Nogo-A protein appears absent from the lesion site itself but seems to accumulate at the proximal and distal boundaries of the lesion (Hunt *et al.*, 2003). It is not clear if the apparent upregulation coincides with increased RGC axonal Nogo-A mRNA levels or whether it can be attributed to oligodendrocytes. Axotomised RGCs sprouting into the lesion site after axotomy appear to weakly express Nogo-A (Hunt *et al.*, 2003).

The role that Nogo-A and its receptor play in inhibiting regeneration is not fully understood. Several studies however, have reported some degree of success in blocking the interaction of NgR with its ligands (Domeniconi *et al.*, 2002; Wang *et al.*, 2002a; Wang *et al.*, 2002b). In the RGC model, delivering a dominant negative form of NgR (NgR^{DN}) by means of an AAV vector, demonstrated that inactivating NgR enhances regeneration in adult RGCs both *in vivo* and *in vitro* (Fischer *et al.*, 2004). However, this approach was only successful when the intrinsic growth state of axotomised RGC was previously boosted via macrophage activation as a result of puncturing the lens (Fischer *et al.*, 2000; Yin *et al.*, 2003). Even though RGCs in adult rats show strong NgR mRNA expression, sole delivery of IN-1 (Caroni and Schwab, 1988a; Rubin *et al.*, 1994) *in vivo* has failed to produce any regeneration in the optic nerve crush model. The combinatorial delivery of IN-1 and BDNF or FGF, on the other hand, increased regeneration after an intracranial optic nerve crush lesion in young rats. Even in this experiment however, a very small proportion of regenerating axons were found at no more than 1.9 mm or 2.5 mm beyond the lesion site when co-delivered with BDNF or FGF respectively (Weibel *et al.*, 1994). A synergistic effect is also reported for IN-1 and CNTF in the intraorbital and intracranial optic nerve crush paradigms (Cui *et al.*, 2004). This study confirmed previous findings proposing that IN-1 delivery alone fails to promote regeneration of axotomised RGCs.

The fact that counteracting NgR on its own does not lead to regeneration is not surprising. NgR undoubtedly plays a key part in the inhibitory nature of the injured CNS but it is unlikely that central myelin components are the only contributing factor. Evidence supporting that this is the case for the injured optic nerve, comes from a set of studies where frozen sections of different developmental stages were used as substrates for RGC axonal growth assays. Tissue sections taken from unmyelinated optic nerves of embryonic (E18-E20) rats, failed to support neurite extension of either neonatal RGCs or both neonatal and adult DRG neurons (Shewan *et al.*, 1993). This observation is replicated *in vivo*, when no axonal regeneration is achieved after optic nerve injury in adult Browman-Wyse mutant rats in which both the oligodendrocytes and CNS myelin are absent. Taken together, these findings prompted Berry and colleagues to suggest that central myelin is not an absolute requirement for regenerative failure of adult axotomised RGCs (Berry *et al.*, 1992). Moreover, *in vitro* results re-enforce the hypothesis made by many research groups that the injured neuron is subject to a complex combination of inhibitory signals, not reproducible *in vitro*. For example, the effect of vaccination in RGC regeneration was demonstrated first by Schwartz and colleagues who showed that vaccinating with myelin derived peptides can lead to neuroprotection for axotomised RGCs but has no effect on fibre regeneration (Fisher *et al.*, 2001). A later study, using transgenic mice overexpressing CNS-reactive T cells, has cast doubt on the original suggestion that activated T-cells are responsible for the vaccine's effect on RGC neuroprotection (Yoles *et al.*, 2001). CNS injury on its own can invoke a T-cell dependent neuro-protective response and it is therefore possible that any neuroprotection afforded by myelin-reactive T-cells may be an indirect effect, mediated by other, non-CNS-reactive lymphocytes (Jones *et al.*, 2002b). In another experiment, optic nerve micro-crush injured animals were injected with spinal cord homogenate. In this study, there was no enhancing effect on RGC survival but axonal regeneration was promoted (Ellezam *et al.*, 2003). Previous studies from the same lab have prompted the authors to form the hypothesis that this effect was mediated by the presence of antigens to myelin associated growth inhibitory proteins. Interestingly, sera from vaccinated animals appeared to have very low levels of antibodies for NogoA, Nogo66, MAG or CSPG (Ellezam *et*

al., 2003). This re-enforces the hypothesis that the inability of RGCs to regenerate through the post injury inhibitory environment involves factors, which are as yet unknown.

In addition to the morphological alterations involving myelin associated growth inhibitory factors taking place in the lesioned optic nerve, the upregulation of extracellular matrix proteins and their poorly understood interactions, are thought to also contribute to the inhibitory nature of the glial scar. CSPGs, shown to be inhibitory to axonal growth in several CNS injury models (Fawcett and Asher, 1999; Logan and Berry, 2002; Sandvig *et al.*, 2004), are secreted by reactive astrocytes by 24 hours after an optic nerve micro-crush lesion (Selles-Navarro *et al.*, 2001). However, the CS-56 antibody used in this study, was non-specific and did not provide any definitive data regarding which member of the CSPG family is upregulated or contributes to the inhibitory environment *in vivo*. A number of specific CSPGs have been implicated in axonal growth inhibition. Neurocan and phosphacan are capable of inhibiting RGC outgrowth, at least *in vitro* (Inatani *et al.*, 2001). NG2, present in oligodendrocyte precursors in the adult mammalian optic nerve (Butt *et al.*, 1999), is reportedly upregulated following optic nerve injury (Chierzi and Fawcett, 2001) and is inhibitory to axonal growth *in vitro* for various CNS neurons (Dou and Levine, 1994; Fidler *et al.*, 1999; Jones *et al.*, 2002a) although *in vivo* its ihibitory nature is uncertain (Rezajooi *et al.*, 2004). Versican, is considered a potential inhibitor of axonal re-growth for the optic nerve that is also expressed by oligodendrocytes and oligodendrocyte precursor cells (Asher *et al.*, 2002).

Nogo, MAG and Omgp as well as CSPGs, seemingly exert their inhibitory effects via the Rho/ROCK pathway and its downstream effectors (McKerracher *et al.*, 1994; Monnier *et al.*, 2003). Rho has been shown to modulate axonal growth by altering cytoskeleton dynamics in the growth cone (Dickson, 2001) and their activation leads to growth cone collapse in RGCs (Lehmann *et al.*, 1999). Targeted inactivation of Rho using the C3 bacterial co-enzyme (Aktories *et al.*, 1989) allows adult RGC axons to grow both *in vivo* after an optic nerve crush and *in vitro* on myelin (Lehmann *et al.*, 1999) or CSPG substrates (Monnier *et al.*, 2003).

Another family of extracellular matrix proteins that has been implicated in the inhibition of adult RGC axonal elongation is the tenascin family (Fournier and Strittmatter, 2001). Tenascin-R (TN-R) inhibits adult and embryonic mouse RGC neurite extension *in vitro*, even in the presence of laminin (Bates and Meyer, 1997), while it persists in the adult mouse lesioned optic nerve for at least 63 days post lesion (Becker *et al.*, 2000). In contrast, tenascin-R quickly disappears from the regenerating optic nerve of salamanders (Becker *et al.*, 1999). A phosphacan-related ligand that co-localises with tenascin-R in the retina and optic nerve of adult rodents has been identified as a proteoglycan that, when offered as a substrate *in vitro*, neutralises the growth cone repulsion induced by TN-R (Xiao *et al.*, 1997). Recently, Te38, a proteoglycan that co-purifies with tenascin C (Bartsch *et al.*, 1992), was also found to inhibit neurite extension *in vitro* (Henke-Fahle *et al.*, 2001).

Each glial cell type in the developing optic nerve i.e astrocytes oligodendrocytes and their precursors, differentially express members of the semaphorin family (Goldberg *et al.*, 2004). Sema-5A, expressed by oligodendrocytes and their precursors in the developing optic nerve (Oster *et al.*, 2003), can induce growth cone collapse in both embryonic, postnatal and adult RGCs when presented as a substrate *in vitro*. Moreover, RGC axons extend 50% further on P8 optic nerve explants in the presence of sema-5A blocking serum (Goldberg *et al.*, 2004). After a complete optic nerve transection in adult rats, sema-3A is quickly upregulated, reaching its peak at 3 days post lesion, and then gradually declining to normal levels. Intravitreal delivery of a sema-3A neutralising peptide, 24 hours after axotomy, appeared to rescue the majority of RGCs from death (Shirvan *et al.*, 2002). Semaphorins appear to also play a role in the inability of RGCs to regenerate and therefore present good targets for regeneration experiments.

6.1.4 Regeneration & neuroprotection in axotomised RGCs

A variety of different approaches have shown some degree of success in encouraging axotomised RGCs to regenerate. The following sections review the reported application of these techniques with regards to the injured optic nerve paradigm, placing emphasis on the use of neurotrophins.

6.1.4.1 Peripheral nerve grafts

A peripheral nerve (PN) graft, transplanted either into the retina (So and Aguayo, 1985; So *et al.*, 1986) or onto the transected optic nerve itself (Berry *et al.*, 1986; Aguayo *et al.*, 1987; Bray *et al.*, 1987; Vidal-Sanz *et al.*, 1987; Berry *et al.*, 1996), is capable of promoting axonal regeneration of axotomised RGCs for considerable distances along the PN graft. A small number of axons succeed in forming functional synapses in the superior colliculus (David and Aguayo, 1981; Keirstead *et al.*, 1989). Berry and co-workers showed that Schwann cells, present in the PN graft, and the trophic molecules they secrete (Friedman *et al.*, 1999; Dezawa and Adachi-Usami, 2000), are the key elements behind this regenerative response (Berry *et al.*, 1988a; Berry *et al.*, 1988b). Later studies revealed that fibroblasts, also present in the PN, secrete a number of trophic molecules, including NGF and BDNF (Acheson *et al.*, 1991), and may contribute to the overall therapeutic effect. There are some reports that suggest that PN fibroblasts are even able to rescue axotomised RGCs (Yip and So, 2000), but there is not enough supporting data to substantiate that claim. Interestingly, re-axotomising the regenerated axons within the PN does not result in significant RGC death (Schuetz *et al.*, 2003). This finding has led the authors of the study to propose that the reduced vulnerability of these RGCs is a property afforded by the Schwann cells engulfing the RGC axons and supporting them with the growth promoting factors they secrete.

Intravitreally transplanted optic nerve segments can also have growth promoting effects on RGC axons. Cho and colleagues have demonstrated that following intravitreal transplantation of either optic nerve or sciatic nerve segments, similar numbers of RGC axons can regenerate along a PN graft, anastomosed to an intraorbitally transected optic nerve (Cho *et al.*, 2001). When a sciatic and optic nerve segments are co-transplanted intravitreally, there appears to be a synergistic effect on the numbers of regenerating RGC axons, compared to the effect obtained following either graft on its own (Cho *et al.*, 1999b). However, only transplanted optic nerve segments were found to increase the survival of axotomised RGCs. On the contrary, optic nerve transplantation alone did not have any effects on RGC sprouting, be it growth promoting or inhibitory, despite

the fact that injured optic nerve slices have been shown to be inhibitory to RGCs *in vitro* (Cho *et al.*, 1999b). Cho and colleagues hypothesise that the mechanism by which grafted optic nerve segments enhance the regenerative effect of the transplanted peripheral nerve, relies on the fact that the optic nerve graft is able to slow down the rate of axotomised RGC death thus leaving more axons receptive to the diffusible growth promoting molecules emanating form the PN graft. This study also showed that *in vivo*, the optic nerve does not impede axotomised RGC survival (Cho *et al.*, 2001).

The experiments described above, have dealt with the regeneration of RGC axons through PN grafts attached to the optic nerve or retina. Berry and co-workers demonstrated for the first time that it was possible for RGC axons to regenerate through the optic nerve of an adult mammal. The optic nerve was crushed intraorbitally and sciatic nerve segments were transplanted into the vitreous body. Over a period of 20 days, a large number of RGC axons had grown across the crush site and up to 3-4mm along the distal optic nerve (Berry *et al.*, 1996). This definitive experiment was the inspiration for the set of experiments that are described in the following sections, and demonstrate even more substantial regeneration of RGC axons.

6.1.4.2 Cataractogenic lens injury

Unaided and significant regeneration appears to be achieved after intravitreal trauma. Simply puncturing the lens or lens capsule (Fischer *et al.*, 2000; Leon *et al.*, 2000) is successful in promoting the survival and inducing axotomised RGCs to survive and regenerate their axons through the inhibitory environment of the lesioned optic nerve. This has been demonstrated following either intraorbital crush (Leon *et al.*, 2000) or transection (Fischer *et al.*, 2001) injuries. When a lens injury is performed 3 days after an optic nerve lesion, 49% of axotomised RGCs still survive 14 days later (Fischer *et al.*, 2000). The extent of axonal regeneration is also impressive. Five weeks after the lesion, up to 30% of RGCs have elongated their axons, from the site of the intraorbital lesion, through the inhibitory environment of the optic nerve and terminated at their retinorecipient

targets in the superior colliculus (Fischer *et al.*, 2001). These regenerated axons function sufficiently to allow modest luminance detection. The exact mechanism by which the lens puncture is able to afford such survival and regenerative properties is not clear. The infiltration into the eye of activated macrophages, a result of puncturing the lens structure, appears to be a contributing factor. Firstly, because activated macrophages secrete a cocktail of trophic factors (Barouch *et al.*, 2001a; Barouch *et al.*, 2001b), and secondly because injection of Zymosan, a potent monocyte activator (Fitch *et al.*, 1999), into the vitreous mimics the effects of a lens puncture even when no axotomy has been performed (Leon *et al.*, 2000; Lorber *et al.*, 2002). Interestingly, if Zymosan is intravitreally injected 3 days after an optic nerve crush, the number of RGC axons regenerating into the optic nerve was even greater than after lens injury (Yin *et al.*, 2003). Somewhat surprisingly, pre-stimulating macrophages with Zymosan 7 days before an intraorbital optic nerve crush lesion is inflicted does not aid RGCs in regeneration, indicating that a factor, additional to those secreted by macrophages, is at play (Yin *et al.*, 2003). Studies of goldfish RGC regeneration revealed that non neuronal cells of the ON secrete a small molecular weight molecule, termed axogenesis factor 1 (AF-1), that can promote significant regeneration of RGCs in culture (Schwalb *et al.*, 1995). A small 15 kDa protein, originating from activated macrophages can induce RGC regeneration *in vitro*, and potentates the effect of forskolin and the mammalian equivalent of AF-1 (Yin *et al.*, 2003).

Compromising the lens also triggers production of neurotrophic factors from Müller glia (Leon *et al.*, 2000) which are known to express growth supporting molecules, such as CNTF *in vitro* (Jo *et al.*, 1999). Interestingly, attempts to block the effects of either CNTF, BDNF or bFGF with neutralising antibodies has no effect in diminishing the beneficial effect of the lens injury (Leon *et al.*, 2000). Lorber *et.al.* proposed that this approach doesn't address the potential combinatorial effect these molecules may have *in vivo* as only one of them was targeted at a time (Lorber *et al.*, 2002). The same authors went on to demonstrate that blocking the trk receptor and CNTF still does not abolish the ability of RGCs to extend neurites in the presence of media conditioned by lesioned lenses.

Whether this holds true *in vivo* is not known as yet but is nevertheless suggestive of the existence of a growth promoting molecule that is neither a neurotrophic factor nor CNTF (Lorber *et al.*, 2002). This line of thought follows the one proposed by Fischer and colleagues (Fischer *et al.*, 2001), who suggested that the survival promoting factors diffusing from the punctured lens epithelium, may account for the neuro-protective effect on axotomised RGCs. The impact of an intravitreal compromise may, to some extent, explain the reported effects of an intravitreal transplantation of nerve segments.

6.1.5 Neurotrophic factors and their effects on axotomised RGCs

Several neurotrophic factors are reportedly capable of promoting survival and/or regeneration in the adult, mammalian lesioned optic nerve with variable degrees of success. Some neurotrophins that appear to have an impact on either survival or regeneration of RGCs *in vitro* appear to be effective only transiently *in vivo*. Here, the effects of each neurotrophic factor on neuroprotection and regeneration as well the various experimental approaches developed for their efficient delivery are discussed.

Factor	Survival of RGCs		Regeneration of RGCs	
	In vitro	*In vivo*	*In vitro*	*In vivo*
BDNF	+	+	-	-
NGF	+	+	+	-
NT3	+	-	-	-
NT-4	+	+	+	-
CNTF	+	+	+	+
FGF	+	+	+	-
GDNF	?	+	+	?
Neurturin (NTN)	+	+	?	?

Table 6.1.5-a: Effects of retinotrophic factors in RGC survival & regeneration.
(Adapted from Yip and So, 2000). + Positive effect, - No effect, ? Unknown.

6.1.5.1 BDNF

BDNF, produced in the superior colliculus (or lateral geniculate nucleus in primates) binds to trkB receptors and is retrogradely transported to the RGC cell bodies in microsomal vesicles (DiStefano *et al.*, 1992; von Bartheld *et al.*, 1996b; von Bartheld, 1998). After intravitreal delivery, exogenous BDNF is transported anterogradely along the RGC axons and released at their targets (von Bartheld *et al.*, 1996a). During development, RGCs exhibit an increased dependency on BDNF for survival as they approach maturity and innervate their targets (Johnson *et al.*, 1989; Thanos and Vanselow, 1989). Delivering purified BDNF in the superior colliculus of neonatal hamsters, protects embryonic RGCs at a time when they are most susceptible to death (Ma *et al.*, 1998). A later study however, has cast a doubt over that claim. Isenmann and co-workers found that delivering BDNF by means of an adenoviral vector to the superior colliculus of neonatal animals did appear to rescue RGCs from death but only in the short term (Isenmann *et al.*, 1999a). This transient neuro-protective effect of BDNF is mirrored in adult axotomised RGCs. *In vivo* studies have established that BDNF can avert apoptotic death of axotomised adult RGCs (Mey and Thanos, 1993; Mansour-Robaey *et al.*, 1994; Peinado-Ramon *et al.*, 1996), but has no effect in axonal elongation (Mansour-Robaey *et al.*, 1994; Cui *et al.*, 1999). The neuro-protective effect of BDNF seems to be independent of the timing of its delivery, with the same number of RGCs rescued when exogenous BDNF is intravitreally delivered either 6 days prior or 5 days after optic nerve transection (Mansour-Robaey *et al.*, 1994). However, BDNF fails to confer long-term survival to RGCs. Even though repeated intraocular injections of BDNF increase the number of viable RGCs, surviving RGC numbers after 6 weeks of treatment resemble those in untreated animals (Mansour-Robaey *et al.*, 1994). In addition to intravitreal injection of purified BDNF, several other methods have been used to induce neuroprotection in injured RGCs. A trk oncogene-containing plasmid injected into the superior colliculus was reported to rescue axotomised RGCs from apoptotic death (Garcia-Valenzuela and Sharma, 1998).

Successful gene transfer and subsequent neuroprotection has also been demonstrated using electroporation *in vivo*. Mo and colleagues report that

delivering mouse BDNF cDNA via electroporation into the eye of adult rats, prevented RGC apoptotic death, with 32.5% still alive 6 weeks after axotomy (Mo *et al.*, 2002). Delivering BDNF to intraorbitally axotomised RGCs by means of an adenoviral vector is also reported to promote survival (Isenmann *et al.*, 1998). However this study does not shed any light on the reasons behind the lack of sustainability of BDNF neuroprotection. Assessment of survival was performed at a fairly early time point (14 days post lesion) thus providing no information regarding the sustainability of this effect. This appeared to have been out of necessity since the vector ceases to express after only 10 days, most likely due to immune system clearance, common with this type of vector (Wood *et al.*, 1996). Virally mediated expression appeared to be non-specific, giving rise to the possibility that the neuro-protective effect observed was a result of the delivered BDNF acting on either RGCs or Müller glia or both. A study published in the same year and using a similar adenoviral vector that targeted the delivery of BDNF to Müller glia for a period of 10 days, demonstrated some short lived RGC neuroprotection (Di Polo *et al.*, 1998). Neurotrophins, including BDNF, have been shown to directly activate intracellular signalling pathways in Müller glia (Wahlin *et al.*, 2000). This temporal nature of neuroprotection can be perceived as an indication that even though BDNF can inhibit the onset of the apoptosis following axotomy (Kermer *et al.*, 1999; Kermer *et al.*, 2000; Klocker *et al.*, 2000), it fails to completely block it (Cui and Harvey, 1995).

The fact that BDNF is expressed in uninjured RGCs and is upregulated following optic nerve axotomy, suggests that endogenous BDNF may contribute to a natural neuro-protective process (Gao *et al.*, 1997). BDNF is reportedly a blocker of the death inducing caspase CASP9 and a potent inhibitor of CASP3 phosphorylation (Kermer *et al.*, 2000; Klocker *et al.*, 2000). Endogenous upregulation of BDNF following axotomy, or exogenous BDNF delivery, can result in indirect adverse effects that hinder rather than promote survival. Firstly, BDNF can enhance the production of nitric oxide (NO) in the retina and its neuro-protective action can be enhanced its production is inhibited (Klocker *et al.*, 1998). However, the BDNF and NO relationship appears to be more complicated since inhibition of NO also causes the down-regulation of growth promoting signals, such as

GAP43, which are known to be upregulated in response to BDNF (Klocker *et al.*, 2001). Klocker and co-workers went on to propose that the mechanism by which BDNF controls the expression of growth promoting molecules involves NO and is separate to that involved in neuroprotection (Klocker *et al.*, 2001). Another possible reason behind this transient nature of BDNF neuroprotection is that after axotomy, the capacity of RGCs to process and respond to this neurotrophin is compromised (Di Polo *et al.*, 1998). After binding to theTrkB receptor, two major signalling pathways are involved in transducing the endogenous and exogenous BDNF signal: MAPK and PI3 kinase/Akt/PKB (Klocker *et al.*, 2000; Nakazawa *et al.*, 2002). Axotomy induces a rapid decrease in TrkB mRNA synthesis, down to 50% that of intact retinas (Cheng *et al.*, 2002). Notably, when TrkB expression is upregulated via AAV gene delivery combined with intravitreally delivered BDNF, 76% of RGCs remain alive 2 weeks after axotomy (Cheng *et al.*, 2002). The same authors report that in this case, signal transduction is carried via the MAPK pathway only. It is therefore possible that when trkB receptors are saturated, a different downstream signalling pathway is involved in signal transduction (Isenmann *et al.*, 2003). A recent study has assessed the expression levels of both full-length (TrkB.FL: 145KDa) and truncated (TrkB.T1: 95KDa) TrkB receptors, following an optic nerve crush and/or exogenous BDNF delivery. Intravitreal BDNF delivery in normal animals has no effect on TrkB.T1 receptors but rapidly causes a significant and prolonged reduction in TrkB.FL protein. Optic nerve crush on its own, also resulted in TrkB.FL down regulation but was only apparent two weeks after axotomy, when 80-90% of RGCs had undergone apoptosis as expected. Combining the two treatments, resulted in down-regulation of TrkB.FL protein, comparable to that observed after the intravitreal delivery of BDNF in normal animals (Chen and Weber, 2004). It is therefore plausible that BDNF hinders its own ability to induce long term neuroprotection by quickly down regulating the levels of its high affinity receptor. Successful RGC survival might rely in combining BDNF with other neuro-protective molecules that utilises a different pathway for signal transduction such as CNTF (Chen and Weber, 2004).

6.1.5.2 GDNF and Neurturin

GDNF (Lin *et al.*, 1993) and Neurturin (Kotzbauer *et al.*, 1996) members of the TGF-β super family, transduce their signals via initially binding to the α subunit of the GFRα co-receptor (Jing *et al.*, 1996) and then activating the transmembrane tyrosine kinase c-Ret receptor (Trupp *et al.*, 1996). Both GFRα1 and GFRα2 are responsible for mediating the signal from GDNF and NTN (Jing *et al.*, 1996; Buj-Bello *et al.*, 1997), even though each seems to demonstrate a preference for GFRα1 or GFRα2 respectively (Lin *et al.*, 1993; Airaksinen and Saarma, 2002). In the uninjured animal, Ret is expressed in the cell bodies and axons of a small proportion of RGCs, while co-localisation studies have revealed that only 14% of RGCs also express Ret even though TrkB is expressed by most RGCs (Jelsma *et al.*, 1993). GFRα1 protein has also been detected in the plasma membrane of cell bodies and axonal fascicles of RGCs as well as Müller glia (Koeberle and Ball, 2002). In situ hybridisation studies show the presence of Ret mRNA and GFRα1 in RGCs while GFRα2 mRNA is localised in the inner nuclear layer (Jomary *et al.*, 1999; Lindqvist *et al.*, 2004).

Following an optic nerve transection, GFRα1 and GFRα2 mRNA levels in the retina increase 2 fold at 14 days after injury compared to normal levels. On the contrary, Ret mRNA levels in the retina decline steadily over 14 days following optic nerve transection and this time course appears to follow that of RGC death (Lindqvist *et al.*, 2004). What happens to mRNA or protein expression of either of these receptors with respect to RGCs is not clear from this report as total retina mRNA extracts were examined and no *in situ* hybridisation or immunohistochemistry was performed. In uninjured animals, GDNF mRNA is localised in the somata and axons of RGCs while protein levels are reportedly very low. Under normal conditions, GDNF mRNA was not detected in the superior colliculus, indicating that GDNF is not a target-derived factor in adult RGCs (Lindqvist *et al.*, 2004). A sharp decline in GDNF mRNA in RGCs following optic nerve transection (Koeberle and Ball, 2002), suggests that the lack of GDNF might pay a role in the onset of RGC death following injury. Notably, the GDNF mRNA decline in RGCs is accompanied by an increased expression of GDNF mRNA in the superior colliculus (Lindqvist *et al.*, 2004).

This study suggests that GDNF might function locally within the retina as part of a neuroprotective mechanism that is initiated in response to injury. Just as is the case for BDNF, this neuroprotective effect appears to be transient, even if prolonged delivery of the factor is achieved. Intraocular (Klocker *et al.*, 1997) or superior colliculus (Yan *et al.*, 1999) administration of purified GDNF is capable of rescuing axotomised RGCs *in vivo* in a dose dependent manner (Koeberle and Ball, 1998; Yan *et al.*, 1999) for up to 14 days after axotomy. Intravitreal transplantation of recombinant fibroblasts expressing GDNF was able to confer some degree of neuroprotection after optic nerve axotomy (Lindqvist *et al.*, 2004). In this study however, transplanting fibroblasts that do not express GDNF still results in neuroprotection. This could mean either that the presence of fibroblasts stimulates macrophage activation or that diffusible factors secreted by the cells themselves can protect axotomised RGCs. Exposure of axotomised RGCs to GDNF by means of an adenoviral vector over 14 days following injury, enhanced the survival of RGCs in a dose dependent manner (Schmeer *et al.*, 2002). However, as this vector does not specifically target neurons, no conclusions can be drawn about whether the neuroprotection afforded by GDNF is a result of its direct effect on RGCs or an indirect effect mediated by other retinal cells, such as Müller glia which also express GFRα1 (Koeberle and Ball, 2002). It has been reported that GDNF can reduce glutamate excitoxicity in axotomised RGCs by increasing the expression of the glutamate/aspartate transporter in retinal Müller glia and astrocytes (Koeberle and Ball, 2002). The hypothesis that GDNF does not exert its effect by directly acting on RGCs might explain why neuroprotection afforded to GDNF-treated RGCs *in vivo*, is not mirrored *in vitro* using purified RGC cultures (Meyer-Franke *et al.*, 1995).

When GDNF and BDNF are simultaneously delivered to the superior colliculus, they appear to rescue axotomised RGCs in an additive and not synergistic manner (Yan *et al.*, 1999). This could indicate that they act through separate mechanisms. Interestingly, the neuroprotective effect of GDNF is dependent on the PI3K pathway while its ability to induce neurite sprouting depends on the MAP pathway (Ho *et al.*, 2000). On the contrary, BDNF neuroprotection involves either the PI3K or ERK pathways, depending on the type of cellular injury and

death signals involved (Hetman *et al.*, 1999). Additionally, sole intraocular administration of neurturin has been shown to rescue 33% of axotomised RGCs from apoptosis at 14 days post lesion (Koeberle and Ball, 2002). In the same study, the combined intraocular delivery of neurturin and BDNF appeared to confer stronger neuroprotection than when combining either neurturin and GDNF or GDNF and BDNF (Koeberle and Ball, 2002). Neurturin, GDNF and BDNF could therefore display an additive effect in promoting survival because they transduce independent signals not only in axotomised RGCs but also in other retinal cells.

6.1.5.3 CNTF

The effects of CNTF are mediated via a receptor complex consisting of the ligand binding, non-signalling, α subunit of the CNTF surface receptor (CNTFRα) and the LIFR and gp130 signal transduction subunits (Stahl and Yancopoulos, 1994). Both CNTF and CNTFRα are involved in the development and differentiation of RGCs (Ip *et al.*, 1993). In the normal adult retina, CNTF immunoreactivity is not detected in RGCs, in contrast to the strong labelling found in astrocytes and weak signal in Müller glia (Kirsch *et al.*, 1997; Ju *et al.*, 1999; Sarup *et al.*, 2004). CNTFRα immunoreactivity is also absent in the normal adult retina while LIFR labelling is present in RGCs and Müller glia (Sarup *et al.*, 2004). One to three days following optic nerve transection, CNTFRα is sharply upregulated in RGCs with LIFR levels remaining unaltered while CNTF expression is increased in Müller glia and astrocytes (Sarup *et al.*, 2004). The CNTFRα upregulation in RGCs following axotomy indicates that they could be responsive to the signals transduced by CNTF. Since CNTF is also upregulated after axotomy but RGC apoptotic death still occurs, it is possible that endogenous CNTF production is not enough to promote regeneration or survival. Therefore, exogenous delivery of CNTF could theoretically overcome this problem.

In vitro studies have demonstrated the ability of CNTF to rescue RGCs from apoptotic death and induce neurite elongation (Lehwalder *et al.*, 1989). However, the ability of exogenously delivered CNTF to enhance survival and regeneration in axotomised RGCs is less clear as the available litterature contains

contradictory reports. In terms of regeneration, repeated intravitreal injections of CNTF but not BDNF, FGF2, NGF, NT3 or NT4, allowed regeneration of intracranially or intraorbitaly axotomised RGCs into a peripheral nerve graft (Cui *et al.*, 1999; Cho *et al.*, 1999a). Cho and colleagues (Cho *et al.*, 1999a) report that the effect of CNTF is dose dependent (Cui *et al.*, 1999). This regenerative ability of CNTF is closely correlated to the observed upregulation of GAP43 (Cho *et al.*, 1999a) and c-Jun (Lu *et al.*, 2003) in the RGCs that regenerate their axons into the periferal graft (Jo *et al.*, 1999). More relevant to the experiments employed in this study, it was shown recently that multiple intravitreal injections of CNTF enable a significant mumber of axotomised RGCs to regenerate their axons into the distal stump of a crushed optic nerve (Cui *et al.*, 2004). However, a single intravitreal injection of CNTF has proven unsufficient to promote regeneration of axotomised RGCs past an optic nerve crush (Leon *et al.*, 2000).

In terms of cell death *in vivo*, intravitreal delivery of recombinant CNTF, enhances RGC survival 2-3 fold, three weeks after axotomy, although this effect diminishes after 7 weeks (Mey and Thanos, 1993; Meyer-Franke *et al.*, 1995). Another group have reported that repeated intravitreal injections of CNTF fail to rescue RGCs from axotomy induced apoptosis following intraorbital or intracranial optic nerve crush (Cui *et al.*, 1999; Cho *et al.*, 1999a). However a recent study from the same laboratory claims that such an injection regime does lead to significant RGC survival, especially when accompanied with elevated levels of cAMP (Cui *et al.*, 2003b). This discrepancy between the two reports may be due to the different method used in assessing surviving RGC numbers, even though the authors do not address this possibility.

More confusion arises when recent gene delivery experiments are considered. In a set of experiments, a secreted form of CNTF (Sendtner *et al.*, 1992), delivered by means of an adenoviral vector, was reported to rescue RGCs from axotomy induced apoptosis but failed to promote regeneration into a peripheral nerve graft (Weise *et al.*, 2000). However, the involvement of Müller glia, which upregulate CNTFRα following axotomy, cannot be discounted given the fact that this vector does not display a neuron specific delivery. Also, intravitreal delivery of this type of vector can cause the activation of macrophages which, since they also secrete

survival factors (Barouch *et al.*, 2001a), would mask the specific effects of CNTF. This could explain why the authors found a significantly higher neuroprotective effect after intravitreal rather than optic nerve delivery. Nevertheless, it has also been reported that CNTF delivery via a self inactivating lentivirus vector to RGCs, also promoted survival (van Adel *et al.*, 2003). It is possible that the inconsistencies in the literature reflect differences between the effects of exogenously delivered CNTF versus endogenously produced CNTF facilitated by gene delivery approaches. After all, intravitreal delivery of purified neurotrophic factors may not be the best mechanism to assess their function since they have a very short half-life and multiple and frequent injections are required. This could further compromise the eye and can make results harder to interpret. The exact mechanism by which CNTF exerts its effects is not fully understood. For example it is not known whether CNTF is secreted from Müller glia and astrocytes after axotomy and translocated to RGCs (Sarup *et al.*, 2004). The fate of CNTFRα in RGCs after axotomy is also elusive. Davis and colleagues hypothesise that CNTFRα, might function as a soluble mediator for CNTF since it can be released from the cells in response to injury (Davis *et al.*, 1993).

6.1.5.4 NGF

Unlike the extensively studied effects of BDNF on neuroprotection of axotomised RGCs, the precise role of NGF as a survival factor for RGCs is unclear, with contradicting reports regarding its efficiency. NGF mRNA is present in adult RGCs and their targets (Lauterborn *et al.*, 1994), while its high affinity receptor TrkA appears to be absent from adult RGCs (Cui *et al.*, 2002). Following axotomy, TrkA levels are upregulated for a brief period (3-5 days post lesion), but rapidly decrease over the three weeks following the lesion (Cui *et al.*, 2002). Repeated intraocular delivery of NGF in neonatal and adult rats can reportedly confer neuroprotection for 7 weeks following intracranial optic nerve transection (Carmignoto *et al.*, 1989). However, several studies have failed to conclusively reproduce this finding either *in vitro* (Cohen *et al.*, 1994) or *in vivo* (Cui and Harvey, 1995). Because of this, exogenous delivery of NGF on its own is unlikely to be beneficial for either survival or axonal regeneration.

6.1.5.5 NT3 & NT4

NT3 and low levels of its high affinity receptor TrkC, have both been localised in the normal adult rodent retina (Allendoerfer *et al.*, 1994; Cui *et al.*, 2002). Following axotomy, TrkC expression is upregulated in injured RGCs and after reaching its maximum after 5 days, high level expression is maintained for the next 3 weeks (Cui *et al.*, 2002). When axotomy is accompanied by an intravitreal transplantation of an optic nerve segment, regenerating RGCs express even higher levels of TrkC (Cui *et al.*, 2002). However, NT3 delivery alone seems to have only a minimal influence on rescuing adult axotomised RGCs from apoptosis *in vitro* (Cohen *et al.*, 1994), and no effect *in vivo* (Peinado-Ramon *et al.*, 1996). Intravitreal delivery of NT3 fails to promote axonal elongation following either an intraorbital or intracranial optic nerve lesion (Cui *et al.*, 1999). NT4 shares its TrkB receptor with BDNF and has been shown to promote the survival of RGCs and neurite outgrowth *in vitro* (Cohen *et al.*, 1994; Bosco and Linden, 1999). The fact that co-administration of BDNF and NT4, even at saturating concentrations does not augment the survival effect of either of these factors alone *in vitro*, indicates that they act via the same TrkB-mediated mechanism (Bosco and Linden, 1999) in rescuing RGCs *in vitro*. *In vivo*, NT4 can rescue axotomised RGCs but the effect is transient even when prolonged delivery is achieved via the use of minipumps (Clarke *et al.*, 1998). As far as axonal elongation is concerned, intravitreal delivery of NT4 is not effective in enabling RGC axon regeneration after intracranial or intraorbital optic nerve transection (Cui *et al.*, 1999).

Even though delivery of either NT3 or NT4 alone fails to avert apoptotic death or regeneration (Cui *et al.*, 1999), both seem to contribute to RGC survival when they are used in combination with other neurotrophic factors such as BDNF, CNTF and FGF. For example, the use of a gene activated matrix to deliver a combination of NT3, FGF2 and BDNF promotes and sustains the survival of axotomised RGCs for a period of 3 months (Berry *et al.*, 2001). Following this study, the authors reported briefly that fibroblasts engineered to express either FGF2, NT3 or BDNF, promote axonal regeneration of axotomised RGCs (Logan *et al.*, 2002). Either of these factors on their own only marginally increased

regeneration with no axons extending past the lesion site. In animals that received all three neurotrophins, GAP43 positive RGC axons were reported to elongate up to 5 mm into the distal stump. However this response seems to disappear after 20 days (Logan *et al.*, 2002). It would therefore appear useful to include NT3 and NT4 among a cocktail of neurotrophic factors in order to assess survival and regeneration of axotomised RGCs.

6.1.5.6 bFGF

Basic FGF (bFGF or FGF2) is a well characterised member of the large family of fibroblast growth factors and is reportedly a potent stimulator of neurite extension in embryonic and postnatal RGCs *in vitro* (Bahr *et al.*, 1989; Dingwell *et al.*, 2000). bFGF transduces its signal via the FGF-1 receptor (FGFR-1), which is required for the correct formation of retinal projections (Brittis *et al.*, 1996). FGF has a high affinity for heparan sulphate proteoglycans (HSPGs) and requires the presence of heparan sulphate in order to activate the FGFR-1 receptor (Lin *et al.*, 1999). In the uninjured adult mammalian retina, bFGF has been localised to Müller glia, the retinal ganglion layer and to the inner and outer nuclear layers (Gao and Hollyfield, 1992; Ohsato *et al.*, 1997). Even though FGFR-1 and HS have been localised to adult RGCs (Sapieha *et al.*, 2003), bFGF is absent from both axons and RGC somata (Kostyk *et al.*, 1994). This is an indication that RGCs have the potential to respond to the presence of FGF. Following axotomy, bFGF is upregulated not only in the GCL and the INL of the retina (Cao *et al.*, 1997b), but also in the lesioned optic nerve itself (Wen *et al.*, 1995) and specifically in the glia around the lesion site (Sapieha *et al.*, 2003). However, bFGF is not upregulated in injured RGCs (Kostyk *et al.*, 1994; Sapieha *et al.*, 2003). The FGFR-1 receptor is only marginally upregulated following a mechanical injury in the retina (Cao *et al.*, 1997b) but no reports as yet detail its fate after RGC axotomy. It has been suggested that Müller cells *in vivo* also respond to the available bFGF by producing even more bFGF, regardless of whether it is released from endogenous stores or delivered exogenously (Cao *et al.*, 1997a). bFGF has been shown to promote survival and neurite extension in adult rat, retinal explant cultures (Bahr *et al.*, 1989). Intravitreal, AAV mediated

delivery of bFGF, has been shown to stimulate only transient survival (up to 2 weeks post injury) and a limited axonal elongation of RGCs (less than 1 mm past the lesion site) after an optic nerve crush (Sapieha *et al.*, 2003). The authors discount the possibility of a macrophage-mediated response as a result of lens damage, though these observations are stated as unpublished. In another report, intravitreal delivery of bFGF was reportedly not sufficient in promoting axonal elongation *in vivo* (Cui *et al.*, 1999). It is possible however that the bFGF levels declined too quickly for it to be effective due to the short half life of bFGF (Culajay *et al.*, 2000; Sapieha *et al.*, 2003). Again, as has been the case with all the neurotrophic factors examined so far, sole bFGF is not sufficient to promote permanent and meaningful survival or regeneration. When used in combination with other neurotrophic factors, its effects are augmented (Berry *et al.*, 2001; Logan *et al.*, 2002) and it would therefore make a worthy component of a group of exogenous delivered factors to injured RGCs.

6.1.6 Rationale

This chapter was designed to test the hypothesis that if the axonal regeneration of RGCs is dependent on the presence of a combination of growth promoting molecules then by transducing injured RGCs to synthesise a cocktail of neurotrophin expressing vectors (rBDNF, mCNTF, rGDNF, rNTN, bFGF), it may be possible to enhance the regenerative response mounted by injured RGCs. This could be measured in terms of regeneration of axotomised RGCs into and through the lesion site. In order to test this hypothesis, a number of different combinations of neurotrophin expressing vectors were tested for their ability to promote axonal regeneration. Their effect was compared to either uninjected animals or animals injected with control vectors expressing GFP or LacZ marker proteins. It was also hypothesised that if there is a correlation between the pattern of transgene expression in RGCs and the severity of IE gene deletions, then utilising a less disabled construct, such as HSV1.RL1^{+}/pR19CMV or HSV1.ICP27^{+}/RL1^{+}/pR19CMV, may lead to a more efficient transgene expression in these neurons. This could in turn translate into measurable difference in both longer and more widespread transgene expression compared to the more disabled HSV1.pR19CMV construct.

6.2 Methods

6.2.1 Vectors used for RGC regeneration experiments

A variety of viruses were used in this study, either to induce regeneration or for the delivery of marker genes to injured or uninjured animals.

Virus	Abbreviation
1764/ICP27$^-$/ICP4$^-$/pR19CMVLacZ	HSV1.pR19CMVLacZ
1764/ICP27$^-$/ICP4$^-$/pR19CMVGFP	HSV1.pR19CMVGFP
1764/ICP27$^-$/ICP4$^-$/pR19CMVratBDNF	HSV1.pR19CMVrBDNF
1764/ICP27$^-$/ICP4$^-$/pR19CMVratNT3	HSV1.pR19CMVrNT3
1764/ICP27$^-$/ICP4$^-$/pR19CMVmurineCNTF	HSV1.pR19CMVmCNTF
1764/ICP27$^-$/ICP4$^-$/pR19CMVratNeurturin	HSV1.pR19CMVrNTN
1764/ICP27$^-$/ICP4$^-$/pR19CMVratGDNF	HSV1.pR19CMVrGDNF
1764/ICP27$^-$/ICP4$^-$/pR19CMVratFGFb	HSV1.pR19CMVrFGFb
1764/ICP27$^+$/ICP4$^-$/RL1$^+$/pR19CMVLacZWPRE	HSV1.27$^+$/RL1+/pR19CMVLacZWPRE

Figure 6.2.1-a: Vectors used in RGC regenration studies.

The construction and characterisation of vectors HSV1.pR19CMVrNT3 (Chapter 4), HSV1.pR19CMVmCNTF and HSV1.pR19CMVrBDNF (Chapter 5) has been described. Constructs HSV1.pR19CMVrGDNF, HSV1.pR19CMVrNTN, and HSV1.pR19CMVrFGFb were produced by Dr B. Haupt (Dept. of Immunology and Molecular Pathology, UCL). Vector HSV1.27$^+$/RL1$^+$/pR19CMVLacZWPRE was constructed by Dr J.A. Palmer in our laboratory.

6.2.2 Injections in the superior colliculus

Adult female Lewis rats (180-250g) were kept under stable anaesthesia using 5% Halothane, delivered by Oxygen throughout the procedure. An incision was made along the midline to expose the cranium. Using a stereotaxic frame to hold a fine drill, the brain was exposed. The dura mater was pricked without crushing the tissue it protects and using a 25 µl Hamilton syringe with a 33-gauge stainless steel needle (RN7762-06), 5-8 µl of the virus was stereotaxically injected at a rate of 0.5 µl/min using a micro pump. The following coordinates were used: rostral-caudal 5.5 mm, medial-lateral 2.0 mm, dorsal-ventral 3.0 mm (Paxinos, 1994). The virus was allowed to diffuse for a further 10 minutes prior to removing the needle. A piece of sterile gel-foam was placed on top of the craniotomy and the skin was sutured.

6.2.3 Injections in the optic nerve

Adult Lewis rats, 6-8 weeks old and weighing between 180-250 g were used for these experiments. The left optic nerve of each animal was exposed by means of a parasaggital 10-15 mm long incision above the eye and halfway between the upper eyelid and the midline. The deep facia, medial to the orbital contents was incised close to the skull, immediately anterior to and in line with the temporal crest. Dissection was continued, using forceps, in the plane between the skull and the hardenian gland. The most anterior part of the fleshy origin of the temporalis muscle, which encroaches into the orbital region, was scraped away, in an anteroposterior direction. The cone of the extraocular muscles was brought into view by further blunt dissection between the hardenian gland and the smaller darker intraorbital lacrimal gland (Kiernan, 1985). Snipping these tissues exposed the optic nerve.

A slit of 1-1.5 mm was made dorsally in the dural sheath of the optic nerve using fine micro scissors. Using a 0.5 ml (29G) insulin syringe needle, the pia mater and arachnoid were carefully pricked to leave a fine slit of about 0.5 mm on the dorsal surface of the optic nerve. A fine glass micropipette, with a tip diameter of approximately 200 μm, was then inserted into the optic nerve proper using a micromanipulator. Using a micro pump holding a 10 μl Hamilton syringe attached to the glass micropipette with fine plastic tubing, 2.5 μl of vector was slowly injected into the optic nerve at a rate of 0.1-0.2 μl/min. After a wait of 5 min, the micropipette was inserted 0.5 mm deeper into the optic nerve whereupon a further 2.5 μl of vector was injected. After a further wait of 5 min, the micropipette was slowly withdrawn, the area was cleaned and the skin was sutured. The integrity of the retinal blood supply was confirmed postoperatively by inspection of the retinal and iris vasculature, which derives its blood supply directly from the ophthalmic artery. An impeded blood supply to the retina can affect the survival of retinal ganglion neurons and interfere with attempts for optic nerve regeneration (Tilgner, 1968).

6.2.4 Optic Nerve Crush

The left optic nerve was exposed as described in the previous section. The dural sheath of the optic nerve was opened with microscisors and the optic nerve was crushed for 20 seconds with fine No.7 curved forcepts ≈1-1.5 mm behind the eyeball. The ophthalmic artery was identified at operation within the optic nerve sheath and was not damaged during surgery. Ocular vasculature was was checked as described in the previous section. Animals were allowed to survive between 3 and 21 days post crush, after which they were transcardially perfused with 4% (w/v) paraformaldehyde (Chapter 2).

6.2.5 Tracing of retinal axons

Three days prior to sacrifice, 5 µl of 1% cholera toxin subunit B (CTB) tracer was slowly injected using a 5 µl Hamilton syringe into the vitreous body of the left eye, avoiding the lens structure. Following perfusion with 4% (w/v) paraformaldehyde, retinas were carefully extracted and examined as wholemount preparations for the presence of GFP or LacZ. Brains were extracted, cryoprotected, cut into 70 µm sections using a freezing microtome and processed for marker gene expression. Optic nerves were embedded in paraffin wax and cut into 12 µm sections, which were then processed for CTB.

6.3 Results

6.3.1 Identification of suitable vector administration route

Based on previous experiments in the spinal cord (Chapter 3), it was decided that the vector most appropriate for transgene delivery to RGCs was HSV1.pR19CMV (1764/ICP27$^-$/ICP4$^-$/pR19CMV). This vector demonstrated the optimum long term delivery to the spinal cord or brain, as transgene expression was significantly more efficient than that achieved with vector HSV1/P2$^-$.pR20.5vhs (Lilley *et al.*, 2001b).

In order to determine the ability of the HSV1.pR19CMV backbone to transduce RGCs, vectors expressing either the LacZ or GFP marker genes were delivered to the superior colliculus. The rationale behind this was to exploit the innate ability of HSV1 to be retrogradely transported to the somata of RGCs and allow them to express the transgene in a specific manner. This route of administration would be ideally suited for assessing the effects of neurotrophins in the injured optic nerve paradigm, as it would exclude any interference caused by the effects of an intravitreal delivery such as a macrophage response or any non-specific tranduction of other retinal cells such as Müller glia. Intravitreal and sub-retinal delivery of HSV1.pR19CMVLacZ and HSV1.pR19CMVGFP was assessed but no transduction of neurons was achieved. A very small number of Müller glia was transduced (no more than 10-20 per retina) via intravitreal injection but not enough to warrant further exploration of this route of administration (results not shown).

Wild type HSV1 does cause a strong immune response when injected into the anterior chamber of the eye (Ganatra *et al.*, 2000). However the HSV1.pR19CMV vector is highly disabled and there is no evidence from our studies in the spinal cord and brain that it is associated with a detrimental immune response. It has also been reported that even for minimally disabled HSV1 vectors, an inflammatory response is initiated when the lens structure is damaged and that this response subsides 3-10 days after delivery (Spencer *et al.*, 2000). No lens injury was inflicted in these experiments. Also, the fact that infected RGCs efficiently express the transgene for at least 20 days, even in the case of LacZ, which has itself been associated with an inflammatory response (Isenmann *et al.*, 2001), is an indication that such a response is not a major factor affecting transgene expression from this vector. 5 x10^6 pfu of HSV1.pR19CMVLacZ were injected into the superior colliculus of a group of animals (n=11). Expression of the transgene was assessed in wholemount retinas and in brain sections.

As shown in Figure 6.3.1-a (panel **A**), the virus enters the RGC axon terminals projecting to the superior colliculus and is retrogradely transported to RGC somata. Transgene expression is evident at 20 days post injection and LacZ

staining can be seen throughout the length of the transduced axons. In the retinal ganglion cell layer, only RGCs are found to express the transgene (Figure 6.3.1-a, panel **B**). This was expected as expression can only occur in the somata of neurons projecting to the superior colliculus where the viral injection took place, which is expected since the vector is incapable of replicating. It cannot therefore exit the RGC somata and infect other neurons or glial cells within the retina itself. Additionally, the vector does not demonstrate a preference for specific RGC morphological classes. Types I, II and III are equally transduced (Figure 6.3.1-c). Examples of all three morphological types of RGCs were successfully transduced in both central and peripheral retina. In terms of examining responses to neurotrophic factors, the fact that all RGC types are transduced can be an advantage as it allows the possibility of targeting the maximum number of RGCs. No difference in the regeneration capacity of the three different RGC types in rodents have been reported but it has been suggested that due to the eccentric organisation of RGCs within the retina, central RGCs are more susceptible to axotomy induced death during an optic nerve crush (Yan *et al.*, 1999). Notably, the co-localisation of transduced RGCs is directly related to the stereotaxic co-ordinates of the injection procedure (Figure 6.3.1-a, panel **C**). In this example, two separate injections of vector into two different sites in the superior colliculus led to two patches of densely packed, transduced RGCs. As RGCs display a topographic organisation throughout the visual pathway, injecting the superior colliculus at different rostro-caudal sites allows the targeting of specific groups of RGCs. This further demonstrates that this vector's characteristics allow for specific neuronal targeting.

The efficiency of transfection achieved with HSV1.pR19CMVLacZ (Figure 6.3.1-a), is replicated when GFP (Figure 6.3.1-b) is delivered by the same backbone. Hoewever, in order to establish a baseline for these vectors in the retinotectal system, it was decided to use the LacZ expressing vectors as they provide a permanent record and allow for easier microscopic examination of transduced cells.

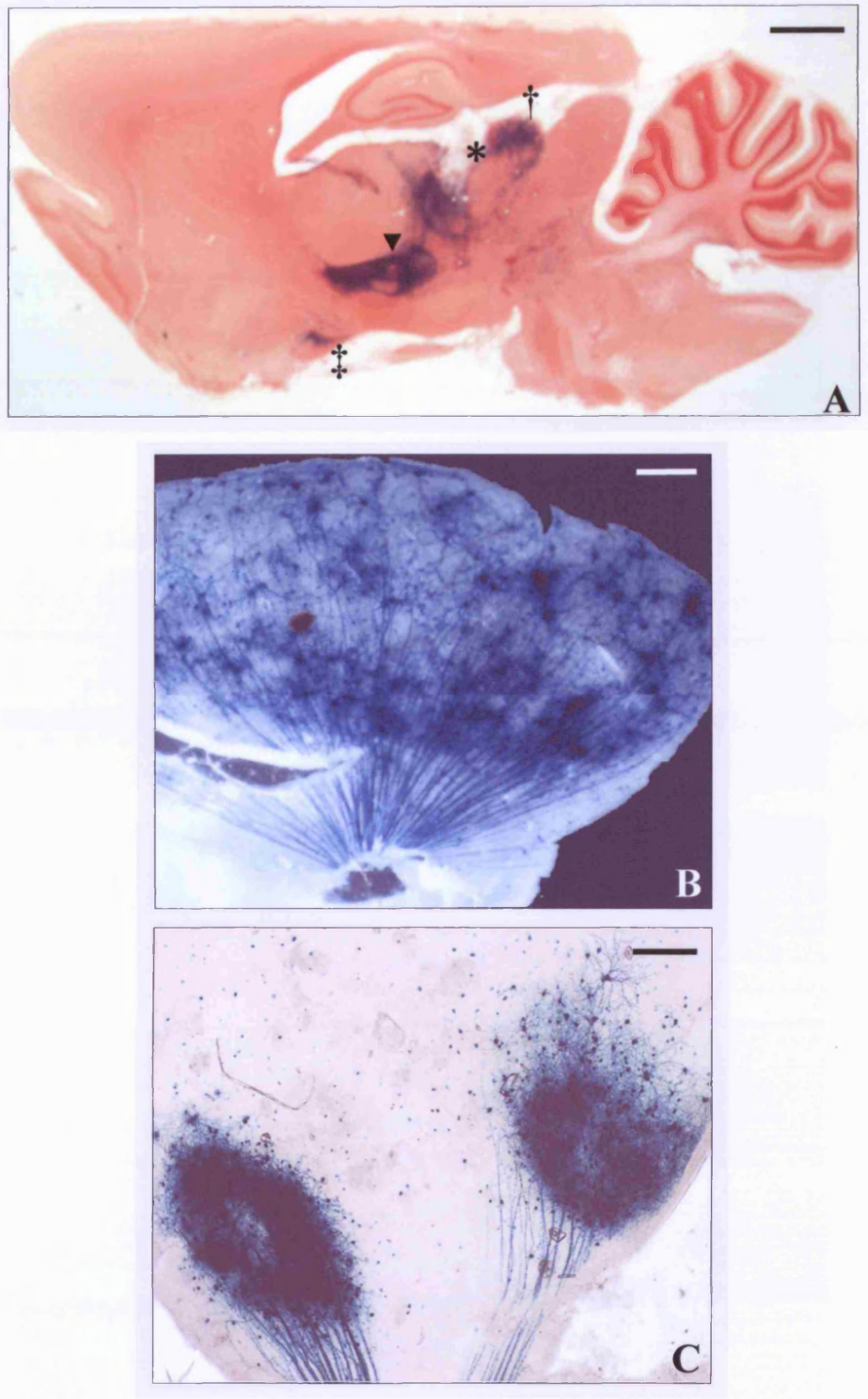


Figure 6.3.1-a: Delivery of LacZ to RGCs via retrograde HSV.pR19CMV transport.

A. Parasagittal, 70μm section, showing the injection site (*) of HSV.pR19CMVLacZ into the rostral superior colliculus (†) of a single animal. RGC axons are filled with LacZ throughout their length and are seen coursing through the optic chiasm (‡) and terminating in the optic thalamus (▼) and superior colliculus (†). **B.** Stable expression in transduced RGCs is observed at 20 days p.i. on a wholemounted retina. LacZ expression can be seen in the cell bodies of RGCs in the ganglion cell layer and their axons radiating towards the optic disc (centre bottom) within the fibre layer. **C.** Retinal wholemount displaying dense segregated patches of transduced RGCs and their axons, demonstrating that topographic delivery to RGCs can be achieved by two separate injections of HSV.pR19CMVLacZ at two different sites within the superior colliculus of the same animal. Scale bars: **A**=2000μm; **B**=500μm; **C**=250μm.

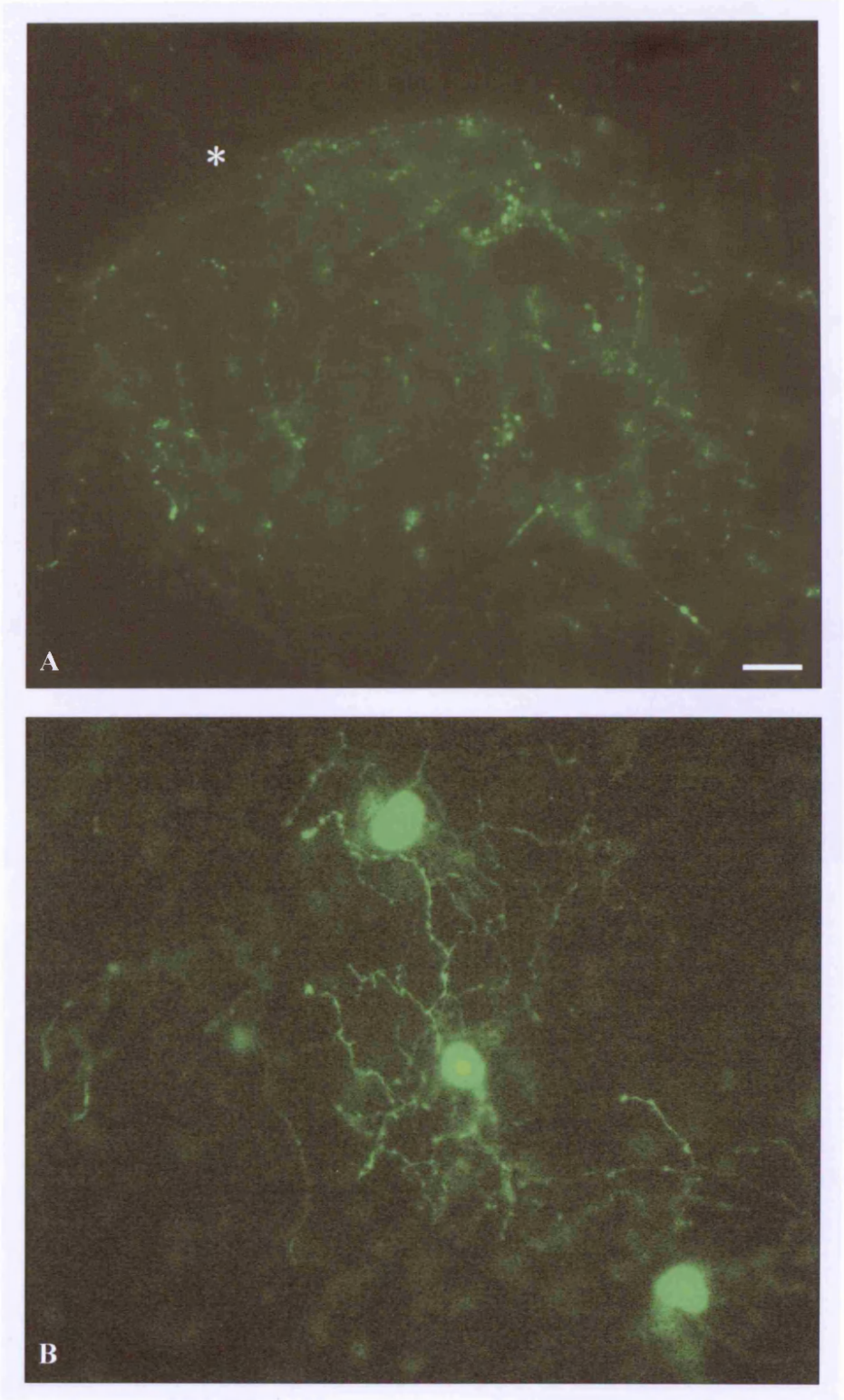


Figure 6.3.1-b: GFP transduction of RGCs via HSV.pR19CMV vector retrograde transport.

A. GFP postive axons close to the injection site (*) of the HSV.pR19CMVGFP vector into the superior coliculus shown in a parasagittal, 70μm, brain section. **B.** shows the origin of these axons as GFP positive RGCs in a retinal wholemount 20 days p.i. Scale bars: **A**=50μm; **B**=20μm.

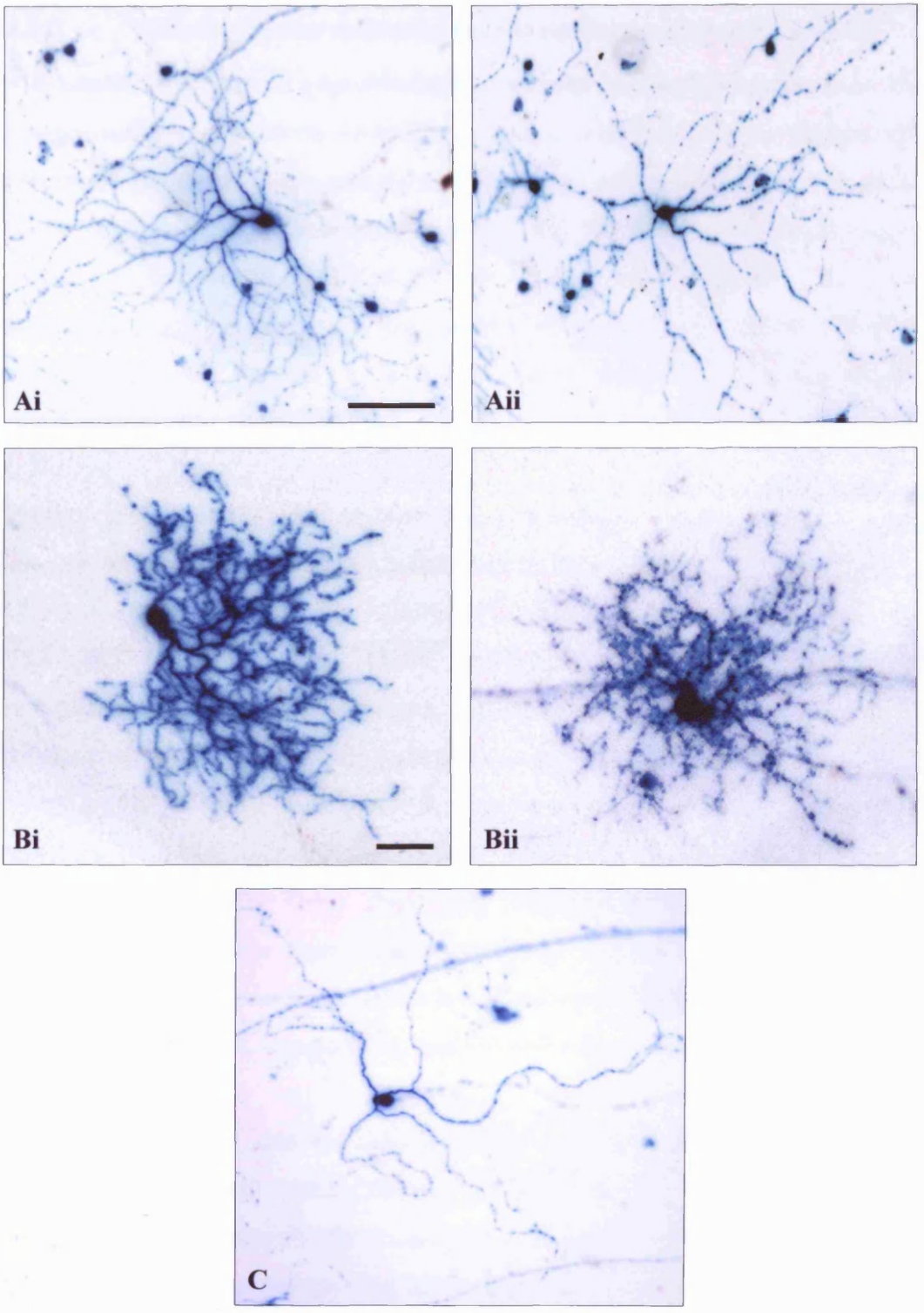


Figure 6.3.1-c: Different classes of RGCs are transduced by HSV.pR19CMV.

All three classes of RGCs were transduced by HSV.pR19CMVLacZ vector injected into the superior colliculus. LacZ expression is strongly maintained at 20 days post-injection. **A** & **B**: Type I RGCs, characterised by their radiating branching pattern of long primary dendrites. **C** & **D**: Type II RGCs recognised by their short primary dendrites with many spiny branches. **E.** Type III RGCs, have fine, long primary dendrites with very few branches. Scale bars: **A**, **B** and **E**=100μm; **C** and **D**=25μm.

6.3.2 Effect of vector delivered neurotrophins on axotomised RGCs

The beneficial effects of a combinatorial treatment in inducing regeneration and neuroprotection of axotomised RGCs has been established in the introductory section of this chapter. The possibility of inducing such an effect on axotomised RGCs when delivering a mixture of neurotrophic and neuroprotective factors by means of the current HSV1.pR19CMV vectors was assessed. This study represents a quantitative rather than qualitative study. Therefore, the effects of administering the mixture as a whole, rather than the individual factors composing it, were examined.

Healthy animals (n=8) were injected with a mixture of vectors, each expressing one neurotrophic factor: HSV1.pR19CMVrBDNF, HSV1.pR19CMVmCNTF, HSV1.pR19CMVrbFGF, HSV1.pR19CMVrGDNF, HSV1.pR19CMVrNT3 or HSV1.pR19CMVrNTN. The cocktail, proportional for each neurotrophic factor, was delivered via multiple stereotaxic injections into the superior colliculus. In order to ensure that any positive effect observed was not a consequence of the vector backbone itself, animals (n=3) were injected with HSV.pR19CMVLacZ vector alone and subsequently treated identically to the cocktail-treated experimental animals. Three days after injection, allowing for retrograde transport and transgene expression to take place, the optic nerve was crushed. Animals were sacrificed 33 days post lesion and optic nerves were examined for anterogradely labelled, regenerating, and GAP43 positive axons.

In some animals that received the cocktail of neurotrophins, a number of RGC axons can be seen regenerating through the lesion site and into the distal stump (Figure 6.3.2-a). In the course of these studies different cocktails were used. Their effects with regards to regenerating axon numbers are varied (Table 6.3.2-a). For example, in one animal (#5), in a single 12 μm longitudinal section of an optic nerve that was crushed following the administration of the neurotrophin cocktail, 24 CTB-labelled regenerating axons were found within the lesion site. In the distal stump, 17 axons were counted at 300 μm past the lesion site, 3 axons at 600 μm and 1 axon at 1 mm. With the assumption that the diameter of the crush optic nerve is about 600 μm, an approximate estimate of the number of regenerating

axons in an entire optic nerve would therefore be 1200 in the crush site, 850 at 300 μm past the lesion site, 150 at 600 μm and 50 at 1mm. Many of the regenerating axons were found in bundles. This may be due to the ability of HSV.pR19CMV to transduce clusters of RGCs that project to specific regions of the superior colliculus. Of course, this estimation assumes a uniform distribution of RGC axons in the optic nerve.

Regenerating RGC axons, found crossing the lesion site into the distal stump, are strongly expressing GAP43. This is further confirmation of their regenerating status. For example in a single 12 μm thick longitudinal section of the optic nerve of one animal (#2, see Figure 6.3.2-b panel **A**), there were 28 GAP43 immunopositive axons in the crush site. Out of these, 14 were found at 300 μm and 1 was found at 1 mm into the distal stump (Figure 6.3.2-b). An approximate estimate of the number of GAP43 regenerating RGC axons per optic nerve would be as follows: 1400 in the crush site, 700 at 300 μm and 50 at 1 mm into the distal stump. Both CTB and GAP43 positive RGC axons displayed a variety of morphological features. They were usually beaded with irregural, non-straight course while some appeared to curve sharply and give rise to short branches (Figure 6.3.2-b). Regenerating GAP43 axons were also found in the pia matter (#1, Figure 6.3.2-b, panel **B**, inset). On the contrary, injection of HSV.pR19CMVLacZ prior to injury, does not promote RGC regeneration (Figure 6.3.2-a). In a single section, only 6 axons were counted as having entered the lesion site and none were found into the distal stump. This is expected as axotomy-induced apoptosis claims the majority of injured RGCs by the end of two weeks.

Animal No	Vector Cocktail								Extent of Regeneration		
	rBDNF	mCNTF	rbFGF	rGDNF	rNT3	rNTN	LacZ	GFP	Crus Site	Distal Stump
1	√	√	√	√			√		*	*
2	√	√	√	√			√		****	***
3	√	√	√	√	√		√		**	*
4	√	√	√	√	√		√		**	*
5	√	√				√		√	****	***
6	√	√				√		√	**	*
7	√	√				√		√	**	*
8	√	√				√		√	**	*
Control Animals (c)										
c1							√		*	*
c2							√		*	*
c3							√		*	*
c4									*	*

Table 6.3.2-a: Neurotrophic factor expressing vector cocktail used.

Total number of animals treated with combinations of neurotrophic factor expressing vector: n=8
Total number of animals that received vector encoding the LacZ marker protein: n=3
Total number of animals not treated with any vector: n=1
All the above animals (n=12) received an optic nerve crush
† Number of regenerating axons found
* 1-5 axons per section
** 6-10 axons per section
*** 11-19 axons per section
**** ≥ 20 axons per section

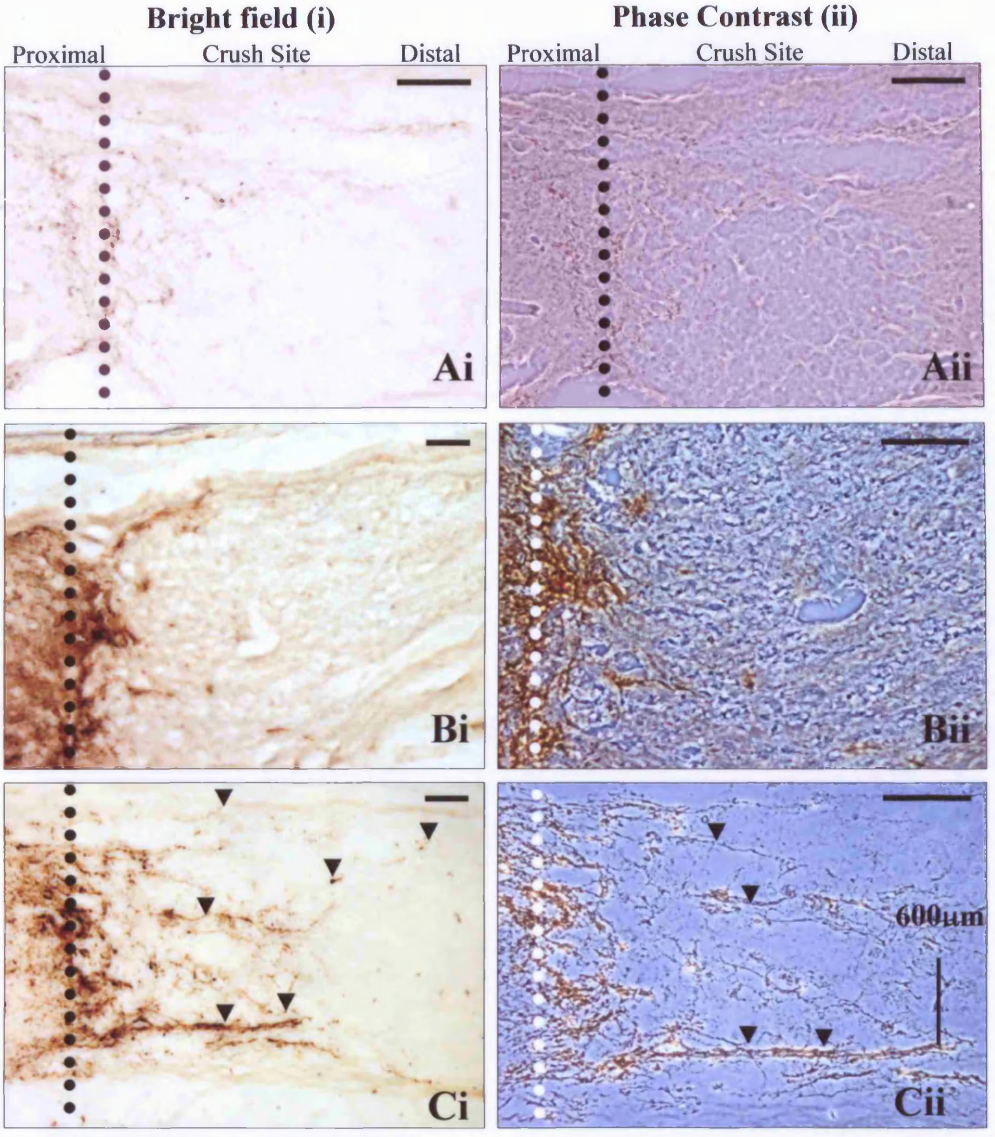


Figure 6.3.2-a: Regeneration of injured RGCs by vector delivered BDNF, CNTF & NTN.

Dark-field (**Ai**, **Bi**, **Ci**) and phase-contrast (**Aii**, **Bii**, **Cii**) images of CTB-labelled RGC axons show minimal regeneration in non-vector injected animals (**Ai** &**Aii**) and in animals injected with the control, LacZ vector (**Bi** & **Bii**). However, in animals treated with a cocktail of neurotrophin vectors (**Ci** & **ii**) there are significantly more regenerating axons (▼) and they have grown further into the lesion site and distal stump than those in control animals. For example, in single 12µm, longitudinal optic nerve sections, the LacZ vector treated animal (**Bi** & **Bii**) revealed only 6 axons in the distal stump, whereas the neurotrophin vector treated animal displayed 24 axons in the crush site and 17 axons in the distal stump. These sections from non-injected, LacZ vector injected and neurotrophin vector injected animals represent animals c4, c2 and 5, respectively in Table 6.3.2-a. Dotted line represents the border between proximal stump of optic nerve on the left and lesion site on right. (*Note:* Even though control animals were injected with HSV.pR19CMVLacZ, it was not possible to react the tissue for LacZ detection, as the product is lost during the paraffin embedding procedure preceding optic nerve sectioning.) Scale bars: **A**=100µm; **Bi** and **Ci**=100µm; **Bii** and **Cii**=100µm.

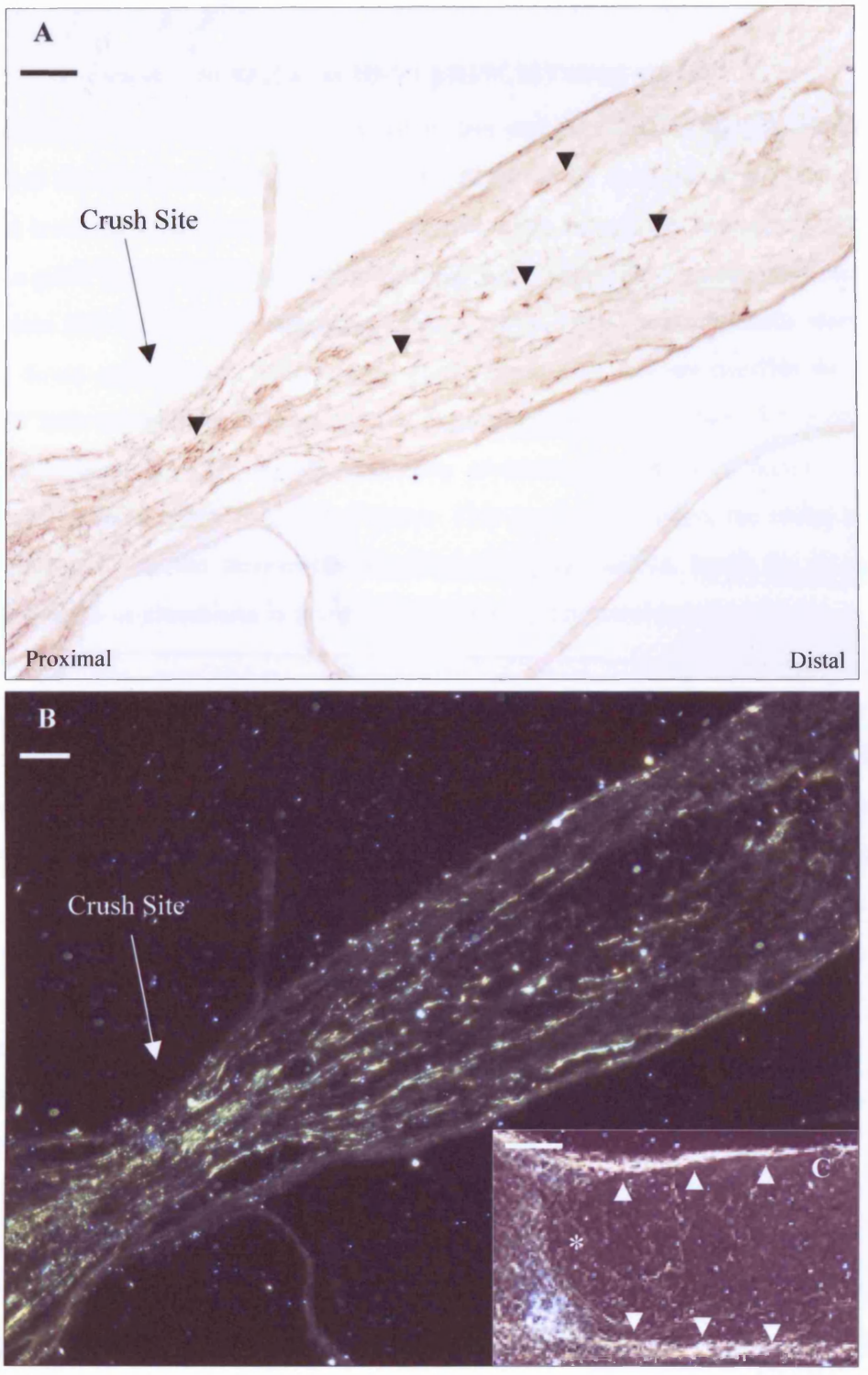


Figure 6.3.2-b: GAP43 expression in regenerating RGC axons.

Bright-field (**A**) and dark-field (**B**) micrographs of a section through the crush site and distal stump of an optic nerve of an animal (#2, Table 6.3.2-a) treated 30 days previously with vectors expressing rBDNF, mCTNF, bFGF and rGDNF. This animal demonstrates substantial regeneration of GAP43 immunopositive RGC axons (arrowheads) through the crush site and up to a distance of 600μm into the distal stump. **C.** shows a dark-field image of GAP43 positive, regenerating RGC axons in the distal optic nerve stump (right), and in the pia mater (arrowheads) in animal #5 in table 6.3.2-a, * crush site. Scale bars: **A** and **B**=100μm; **C**=250μm.

6.3.3 Delivery to RGCs via HSV1.pR19CMVWPRE vector

In addition to the need for both neuroprotective and regenerative signals, another element thought to contribute to successful regrowth of axotomised RGCs is the actual levels at which these factors are present. Even though the vector appears to give a good marker gene expression for both LacZ and GFP, it is not possible to correlate this with actual transgene mRNA levels. Also, in the animals shown here, direct estimation of neurotrophic factor expression was not possible due to either lack of reliable antibodies or unsuitability of the tissue for protein extraction methods. All animals receiving neurotrophin encoding vectors also received an intravitreal injection of tracer. This procedure renders the retina less than adequate for the demonstration of neurotrophin mRNA levels by *in situ* hybridisation or alterations in protein expression by immunohistochemistry.

A new vector was recently developed in our laboratory, which even though less disabled, still showed minimal cytotoxicity and good transgene expression. The vector, termed HSV1.27^{+}/RL1^{+}pR19CMVWPRE, is not deleted for ICP27 or ICP34.5 and incorporates the post transcriptional regulatory element WPRE that has been shown in our hands (unpublished observations) and in other vector systems to enhance gene expression (Zufferey *et al.*, 1999). The Woodchuck Hepatitis Virus (WHV) post-transcriptional regulatory element (WPRE), inserted at the 3' end of the transgene is normally required for the accumulation of wild type viral mRNAs (Donello *et al.*, 1998) and has previously been shown to enhance transgene expression in other vector systems both *in vitro* (Zufferey *et al.*, 1999; Loeb *et al.*, 1999) and *in vivo* (Paterna *et al.*, 2000; Klein *et al.*, 2002; Glover *et al.*, 2003; Hlavaty *et al.*, 2005). Therefore it could, in theory, result in even higher transgene mRNA levels with HSV1 based vector systems. This has been shown to be true in our *in vitro* studies utilising these vectors (manuscript submitted). It was decided to assess whether this combination of backbone deletions would transduce a higher number of RGCs via superior colliculus or direct ON injection and whether the inclusion of the WPRE element would increase transgene expression levels. This vector was easier to propagate in culture and thus higher titres were obtained.

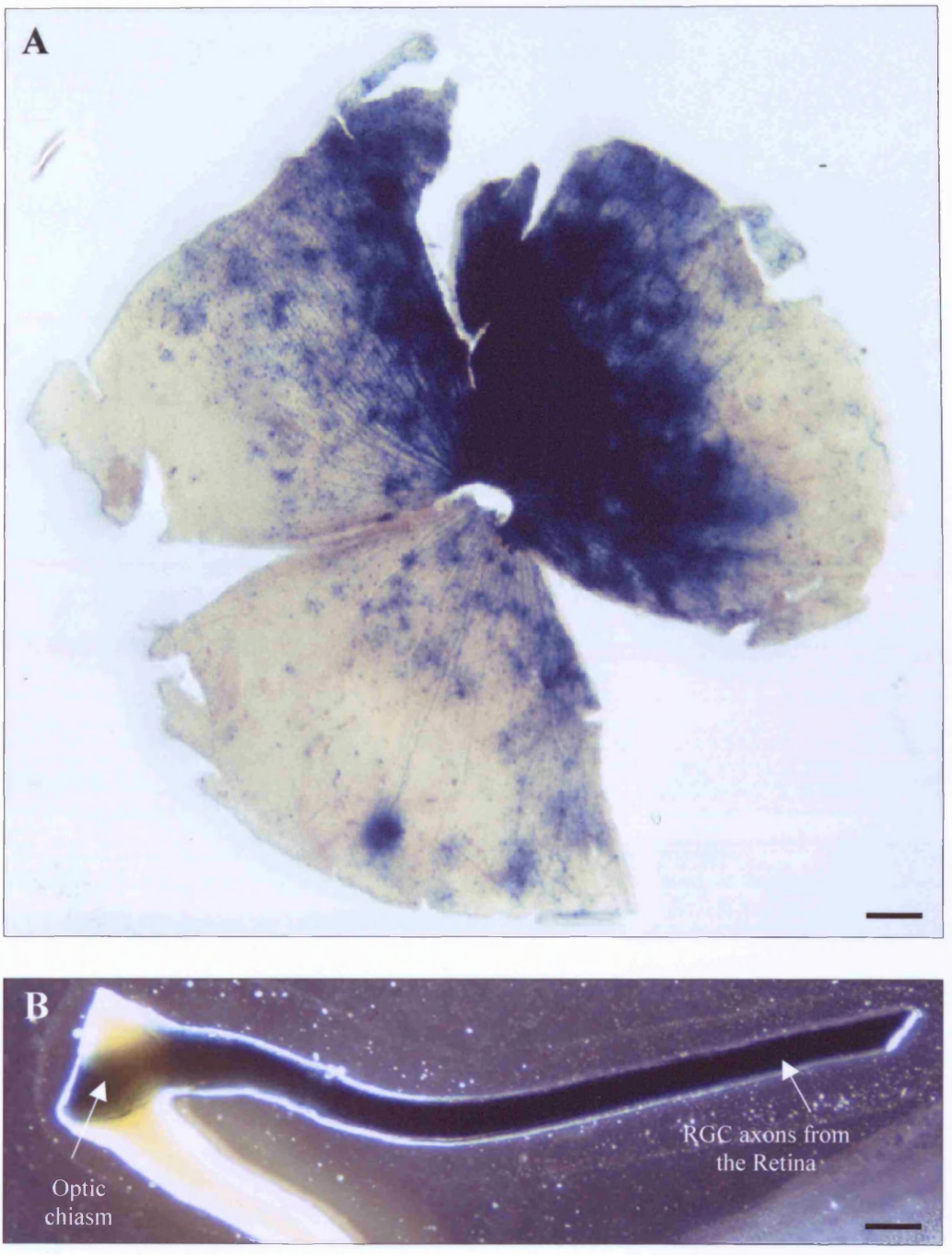


Figure 6.3.3-a: HSV1.27^{+}/RL1^{+}/pR19CMVLacZwPRE mediated transduction of RGCs via a single superior colliculus injection.

Whole mount preparations demonstrating strong LacZ staining in large clusters of RGC somata, dendrites and axons in the retina (**A**) and in RGC axons throughout the optic nerve and chiasm (**B**). Scale bars: **A** and **B**=500μm.

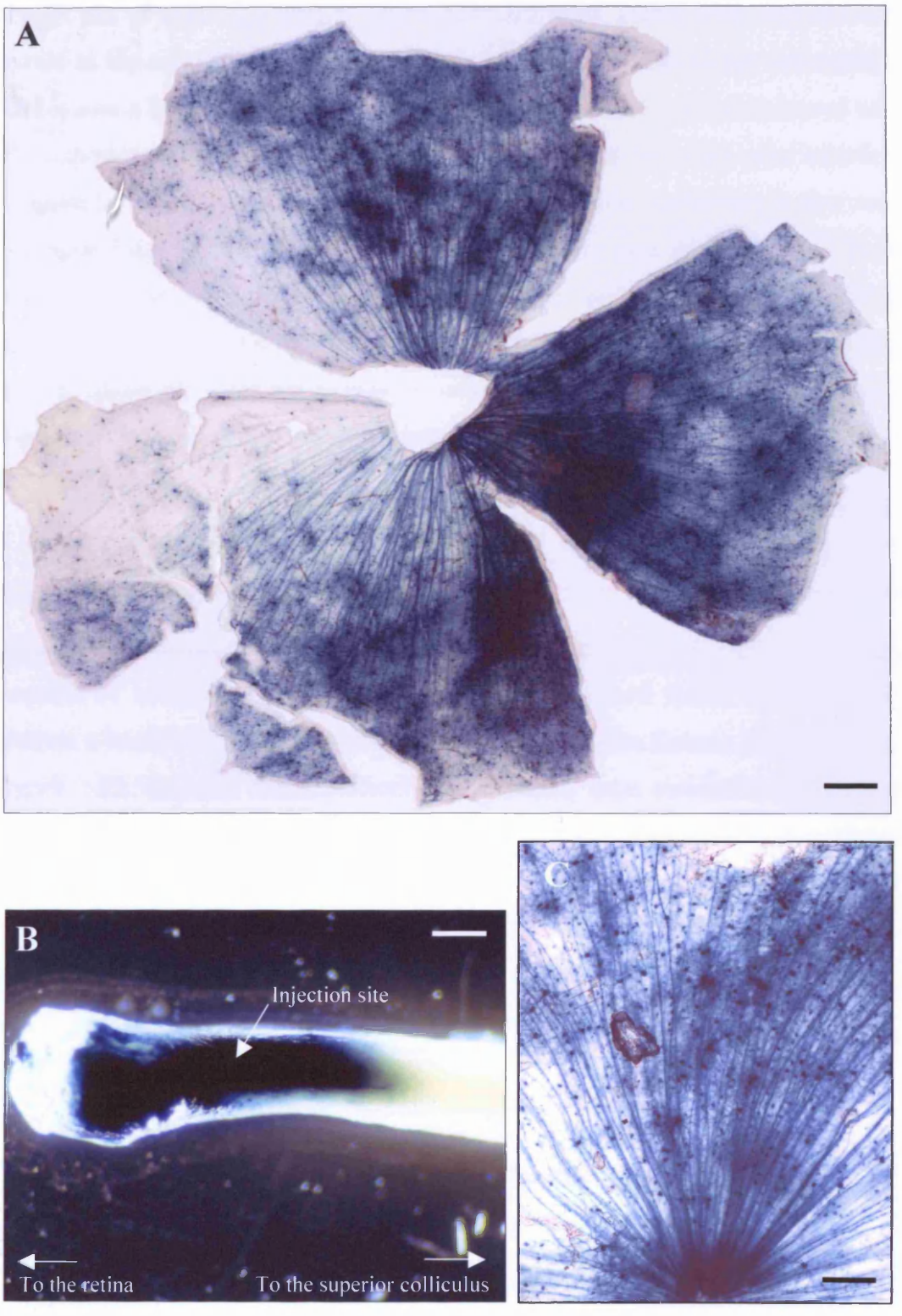


Figure 6.3.3-b: Transduction of RGCs via a single HSV1/27^{+}/RL1^{+}/pR19CMVLacZ$_{WPRE}$ optic nerve injection.

Wholemounted retina (**A**) demonstrates substantial numbers of LacZ positive RGCs and axons expressing transgene (seen at higher magnification in **C**). LacZ positive RGC axons fill the region of the injection site in the optic nerve in the wholemount preparation (**B**). Scale bars: **A**=500μm; **B**=250μm; **C**=250μm.

$2x10^7$ pfu of HSV1.27$^+$/PRL1$^+$ pR19CMVLacZWPRE vector were administered either in the superior colliculus or directly into the ON. This vector successfully transduces a large number of RGCs within the retina when it is administered into the superior colliculus with strong expression achieved one week after injection (Figure 6.3.3-a). In the case of direct ON administration, expression is also very strong at 7 days post injection (Figure 6.3.3-b). By the time this study came to an end, a new set of vectors utilising this backbone and each expressing one of BDNF, CNTF, NT3 and GDNF were produced and later shown to be biologically active. However, it was not possible to utilise them *in vivo* due to time constrains and they are therefore not presented here.

6.4 Discussion

This study demonstrates for the first time that HSV1.pR19CMV vectors are capable of inducing a regenerative effect in axotomised RGCs when used to deliver a combination of neuroprotective and regenerative factors. No other HSV vector with this level of disablement has reportedly been successful in inducing RGC regeneration *in vivo*. Even though this result is encouraging as it confirms that a combinatorial delivery promotes re-growth and rescue, it sheds little light on the role that each of the neurotrophic factors used play in RGC neuroprotection and regeneration. This should be the natural progression of this study and the HSV1.pR19CMV vector can be effectively used to explore their interactions.

The fact that expression is allowed to proceed for three days prior to inflicting the optic nerve crush may be enough time to prime RGCs with sufficient amounts of survival and regeneration promoting factors so that they have an enhanced growth capacity once the injury is inflicted. The enhanced survival promoting effects of simultaneously delivering BDNF, GDNF and NTN (Koeberle and Ball, 2002) combined with neuroprotective and regenerative signals of CNTF, bFGF and NT3 allows for a favourable regeneration environment. Since the regeneration shown here is achieved at 33 days after injury, it is logical to suggest

that neuroprotection was successful to some degree. With hindsight, the numbers of surviving RGCs should have been accurately determined so that the actual rescue effect can be compared to that reported for each of these factors. Similarly, the exact number of regenerating RGC axons in each treated optic nerve should also have been established and compared to that obtained in the literature. However, as already stated, this study was focused more on the ability of these vectors to induce an overall biological effect when delivered in combination, since previous attempts at spinal cord regeneration delivering a single neurotrophic factor proved fruitless.

Disappointingly, only a small number of animals yielded any respectable RGC axon regeneration following NT-vector administration. This could be due to a number of reasons. Firstly, from the preliminary experiments conducted using marker gene delivery (LacZ or GFP) we found the stereotaxic injection into the RGC terminals within the superior colliculus to be unreliable. This may be due to the fact that even though the superior colliculus is a large area, its retinorecipient layer is very thin. It is therefore technically difficult, even with a good stereotaxic frame and co-ordinates, to reliably localise the site of an injection to this layer. Multiple injections into the superior colliculus generally yielded higher numbers of transduced RGCs. Secondly, the optic nerve crush procedure used may not be as reliable as one would like. For example, too much pressure applied via the forceps could yield a crush site that resembles a transection. This would not be as conductive to regrowth along the distal stump as a crush. On the other hand it is necessary to apply adequate pressure as an incomplete crush may result in some RGCs being spared axotomy. However, it is unlikely that this was the case in the vector treated animals that did demonstrate robust axonal regeneration. Spared RGC axons have a smooth morphology, follow a straight course through the optic nerve and more importantly they are present throughout the length of the optic nerve. The regenerating axons found in the neurotrophin treated animals in this set of experiments were beaded and branched with a highly irregular course. They were also of limited length and never observed more than 1 mm along the distal stump of the optic nerve. Thirdly, the standard method of intravitreally injecting the CTB tracer may not be as reliable as it is easy for it to leak out from

the puncture wound in the limbus, thus limiting the amount available for uptake by RGCs. We have tried to overcome this problem by processing alternate sections for both CTB and GAP43 immunohistochemistry. However, this was not performed for every animal.

In terms of inducing regeneration in the already injured optic nerve, which would be of more interest in terms of clinical relevance, further developments in the design of the HSV.pR19CMV vector have presented the possibility of directly targeting the optic nerve itself. By utilising a newly developed vector that is not disabled for the expression of ICP27 or ICP34.5 and incorporates the use of a WPRE element, this vector allows for not only higher expression of transgenes in the retina but also allows targeting of the optic nerve milieu itself. This would allow even more direct targeting of the vector to only RGCs rather than other neuronal populations that may inevitably be transduced when the vector is delivered in the superior colliculus. It would be interesting to assess the differences that the WPRE element makes to the protein levels of these factors and more importantly how these correlate to their biological effect.

SUMMARY

In summary, this chapter demonstrates that the viral mediated transduction of RGCs with a combination of survival (rBDNF, rGDNF and rNTN) and regeneration (mCNTF, bFGF and rNT3) promoting signals enhances the regenerative response mounted by injured RGCs. This response is accompanied by evidence of GAP43 upregulation and RGC axonal elongation into the lesion site. In addition, the use of less disabled constructs such as HSV1.RL1^{+}/pR19CMV or HSV1.ICP27^{+}/RL1^{+}/pR19CMV leads to a more efficient transgene expression in these neurons in terms of RGC numbers transduced and length of transgene expression.

CHAPTER 7.0

EFFECTS OF INTERLEUKIN 10 ON VECTOR MEDIATED TRANSGENE EXPRESSION IN DRG NEURONS

7.1 Introduction

In previous chapters, (Chapters 3, 4 and 5) efforts have been concentrated at targeting spinal cord motor neurons via a direct stereotactic injection. Even though this technique does result in widespread neuronal transduction it is a relatively traumatic practice that may further exacerbate injury in the already compromised CNS. An alternative technique that would allow us to circumvent this problem would be to deliver potentially therapeutic molecules via peripheral nerve inoculation thus exploiting the innate ability of HSV1 to be retrogradely transported to neuronal cell somata. Such an approach has been previously attempted in our laboratory (Perez *et al.*, 2004) when the minimally disabled vector 1764/pR20.9UL43 (HSV1.pR20.9/UL43) was compared to the highly disabled HSV1.pR19CMV vector. These experiments demonstrated that even though both vectors are capable of transducing DRGs and anterior horn motor neurons, transduction ceases relatively quickly (Palmer *et al.*, 2000; Perez *et al.*, 2004).

One of the potentially contributing factors to the termination of transgene expression may be an immune response to the virally infected cells (Perez *et al.*, 2004; Simmons *et al.*, 1992). This probability would explain the transgene expression patterns observed with either HSV1.pR20.9/UL43 or HSV1.pR19CMV vectors. HSV1 has innate machinery for evading the host immune responses and inhibiting apoptotic signals within the infected cell. However, the vectors used for delivery to the CNS are highly disabled and even though this may be beneficial in terms of reducing cytotoxicity, it also implies that its mechanisms for immune evasion are compromised. Even though these vectors can support transgene expression in the CNS for at least three weeks, possibly due to the more immunocompromised nature of the CNS milieu this is not the case when the same vectors are used to deliver transgenes to the PNS. Transgene delivery is not as extensive while expression is minimal after two weeks. The same pattern is noted when the less disabled vector HSV1.pR20.9/UL43 is used to transduce DRG and anterior horn motor neurons following a footpad inoculation. Again, transgene expression is notably strong at the 2-day time point but it is not sustained past the two-week time point. Given

the fact that the immune response in the PNS is stronger than that observed in the CNS, it is possible that the HSV1.pR20.9/UL43 vector, even though capable of establishing latency in infected cells and initiating transgene expression, it is at the same time unable to evade the PNS immune response triggered by the infection itself. The observed lack of transgene expression could therefore be perceived as the result of losing the latently infected cells altogether or elimination of some of the inoculum as a consequence of an immune and inflammatory response.

7.1.1 HSV1 infection and the host immune response

The first line of defence following infection with HSV1 involves the activation of the innate immune response. This response involves the production IFN α or β and subsequent recruitment of natural killer (NK) cells as well as other antigen presenting cells (APCs) such as dendritic cells (Biron, 1999). Initial recruitment of innate immune response participants as well as the nature and strength of the subsequent adaptive immune response is shaped through leukocyte secreted cytokines (Ramshaw *et al.*, 1997). Additionally, a rough distinction must be made in the nature of the host immune response between the acute and latent stages of infection with HSV1. The mechanisms responsible for resolving HSV1 infections from the skin differ to those employed in clearance from the PNS. They involve different types of cells and the host-virus relationship is balanced by elaborate virus evasion strategies.

The host immune response during the brief, acute phase of HSV1 infection is primarily innate, involving $CD4^+$ T cells and a Type IV hypersensitivity reaction (Nash *et al.*, 1987). $CD4^+$ T cells are the first to infiltrate the site of infection in response to Major Histocompatibility Complex (MHC) Class II protein-antigen complex recognition on APCs which in turn triggers their differentiation into cytokine secreting effector cells (Mikloska *et al.*, 1998). Activation of naïve T-cells requires the simultaneous presence of MCH Class II-antigen complex and activation of co-stimulatory signals by APCs (Male *et al.*, 1996). Effector cells of the $CD4^+$ class, can be further divided into two populations, either T-helper 1 (Th1) or T-helper 2 (Th-2), depending on the profile of cytokine expression

(Keane and Hickey, 1997). $CD4^+$ Th1 cells secrete IL2, IFNγ and TNFα that activates $CD8^+$ T cells, also known as cytotoxic T-lymphocytes (CTLs). $CD8^+$ T cell receptor recognises and facilitates the lysis of cells that present MHC class I antigens leading to clearance of infection. They do so by triggering an autocatalytic suicidal cascade within the target cell, involving the Src-family of protein kinases. The resulting modification of cellular processes such as calcium mobilisation and the activation of the Ras pathway causes DNA fragmentation and an apoptosis-like death (Mullbacher and Ada, 1987).

HSV1 infection leads to a sharp increase in cytokine and chemokine levels, which are important for the recruitment of cells that initiate an effective immune response. Induction of RANTES expression, the main chemokine-induced by macrophages during a primary infection, is triggered by ICP0 *in vitro* (Melchjorsen *et al.*, 2002). In addition, cellular IFN induces the double-stranded RNA-activated protein kinase R (PKR) (Melchjorsen *et al.*, 2002). PKR is a central factor in the up regulation of TNFα, IL-6, and RANTES, since expression of a dominant-negative mutant form of PKR strongly inhibits production of these cytokines in HSV1 infected macrophages (Melchjorsen and Paludan, 2003; Paludan and Mogensen, 2001). T lymphocytes require the presence of two signals for proliferation, induction of effector functions and initiation of a maximal T-cell response (Male *et al.*, 1996). The first signal depends on the binding of the $CD8^+$ or $CD4^+$ T cell receptor (TCR) to MHC class I or II respectively on APCs. The second signal is antigen independent and relies on the interaction between the B7-costimulatory molecules expressed on APCs and their ligands present on T cells (Singh *et al.*, 2003). Even in the presence of the first signal, absence of co-stimulation by B7 isomers, B7-1 (CD80) and B7-2 (CD86) results in T-cell antigen specific clonal anergy which renders T cells incapable of responding to antigen (Bretscher, 1992; Mueller *et al.*, 1989). Unlike the humoral response, the T cell-mediated immuno-protection is mediated by cell disruption (Male *et al.*, 1996; Mullbacher and Ada, 1987). It is therefore not surprising that cytotoxic responses are tightly regulated in both PNS and CNS. The inflammatory infiltrate observed during the initial stages of infection is composed of monocytes, NK cells, Th1 $CD4^+$ and γ/δ $CD8^+$ T cells (Liu *et al.*, 1996). NK cells and $CD8^+$ T

cells secrete IFN-γ which can prevent host cell apoptosis by inhibiting HSV1 replication (Halford *et al.*, 1997). IFNγ, also induces the extravasation of polymorphonuclear (PMN) leukocytes (Liu *et al.*, 1996) and activation of NK cells and macrophages (Barcy and Corey, 2001) that phagocytose viral particles in a CTL-independent and non-specific manner, primarily through their C3 receptors for complement formed via the alternative pathway (Keane and Hickey, 1997; Male *et al.*, 1996). IFNα results in production of defective virions (Munoz and Carrasco, 1984) and direct inhibition of viral replication due to a block in α and β viral gene transcription (Straub *et al.*, 1986). Cytokines also stimulate the clonal differentiation of B-cells. B-lymphocytes, activated by the $CD4^+$ Th1 effector cells, become plasma cells and secrete antibodies specific to viral antigens. These recognise epitopes on the virus required for infection and by binding to them via their Fab region, render them neutral. B-lymphocytes, can also recognise epitopes through their specific immunoglobulin (Ig) receptors present on their cell membrane. Once bound, the Ig-viral antigen complex is engulfed, partially digested and the antigenic portion is displayed on its cell membrane as a complex with the MHC Class II protein. IgG and IgM class antibodies are also crucial in complement fixation via the classical pathway (Male *et al.*, 1996).

Effector	Cytokine	Effect
Th1	IFN-γ	• Enhances MHC class I and II expression. • Increases macrophage phagocytic activity by inducing Nitric Oxide synthase.
	TNFα	• Produced by B-cells/astrocytes in response to viral infections & IFNγ
	IL-2	• Influences growth of oligodendrocytes, macrophages and B-cells. • Initiates activated T-cell clonal expansion.
Th2	IL-13	• Regulator of B-cell and monocyte activity.
	IL10	• Inhibits cytokine synthesis by activated Th1 and NK cells. • Down regulates MHC class II expression on macrophages. • Synergistically with TGFβ causes IgA production by B-cells.
	IL-6	• Promotes growth and maturation of B-cells. • Enhances the secretion of IgG by B-cells.
	IL-5	• Induces the expression of IL-2 receptor on B-cells • Enhances the production of IgA from B-cells.
	IL-4	• Promotes class switching of B-cells to IgE (contributes to inflammatory reactions) & augments IgG production. • Enhances growth of T-cells & B-cells.

Table 7.1.1-a: Expression of cytokines by CD4+ effector cells and their effects.

Adopted from Male *et al.* 1996.

7.1.2 Latent stage of infection & the PNS immune response

The CNS and PNS are shielded by the blood-brain (BBB) and blood-nerve (BNB) anatomical barriers respectively. These barriers along with the lack of regular lymphatic drainage, the absence of intraneural lymphatic channels and the atypical expression of MHCs (Oldstone, 1991), afford an immune privileged environment (Theil *et al.*, 2003). However, both BBB and BNB are permeable to activated T cells (Allt and Lawrenson, 2000; Hickey, 2001) which constitute the main immune surveillance mechanism for both the CNS and PNS . The ability of nervous tissue to tolerate an immune response is due to an active process that tightly controls its nature, quantity and quality with priority focused on preserving organ integrity. Unwillingly, this mechanism also provides a survival advantage to the virus present within neurons.

Latent infections in the PNS are associated not only with the expression of LATs but also with low level, constant expression of viral immediate-early (IE) genes such as ICP4 and thymidine kinase (TK) (Kramer and Coen, 1995). The establishment of a persistent, latent infection in sensory ganglia instigates an adaptive immune response (Simmons and Tscharke, 1992) involving primarily γ/δ TCR $CD8^+$ T cells (Liu *et al.*, 1996). This response, further enforces latency and controls infection via non-cytolytic mechanisms based on cytokine secretion (Guidotti and Chisari, 2001). It is these responses that cause latent HSV1 infections to be coupled with a low grade, chronic inflammation. The persistent infiltration of γ/δ TCR $CD8^+$ T cells is accompanied by high levels of IFNγ expression (Cantin *et al.*, 1995; Liu *et al.*, 1996), altered expression or distribution of GAP43 (Martin *et al.*, 1996), upregulation of nitric oxide synthetase (Koprowski *et al.*, 1993; Meyding-Lamade *et al.*, 1998) and upregulation of TNFα (Theil *et al.*, 2003) which synergises with IFNγ to further block HSV1 replication (Feduchi *et al.*, 1989; Paludan and Mogensen, 2001). In response to secreted chemokines, macrophages also infiltrate the infected area. Studies in the HSV1 latently infected human trigeminal ganglion neurons (Lodge and Sriram, 1996) demonstrated the presence of very high levels of RANTES in macrophages (Melchjorsen *et al.*, 2002). RANTES is thought to play a crucial role in the recruitment of other immune cells to the site of infection.

Persisting $CD8^+$ T cells can interact with latently infected neurons or macrophages (Khanna *et al.*, 2003). Indeed, $CD8^+$ T cells are found in high numbers around the cell bodies of latently infected neurons but they do not interact directly with them. They do however interact directly with satellite cells (SC) (Liu *et al.*, 1996) which have been shown to upregulate MHC Class I expression (Pereira *et al.*, 1994) in response to HSV1 infection. SCs are also thought to play a role in neuroprotection in response to HSV1 infections even though the exact mechanism by which they do is still unclear. SCs resemble schwann cells in their phagocytic capacity (Wilkinson *et al.*, 1999) and they are notoriously difficult to differentiate from other immune system infiltrating cells (Elson *et al.*, 2003). SCs are found to contain empty or incomplete HSV1 virions, suggesting that they undergo an abortive infection (Dillard *et al.*, 1972). Interestingly, SCs proliferate in response to infection by HSV1 and almost double in numbers around the infected sensory ganglia. Several hypotheses have tried to address the reasons behind this response to neuronal infection. One hypothesis is that it is initiated in response to direct stimulation by viral factors (Elson *et al.*, 2003). The only evidence-supported explanation so far is that normally, SCs are kept under inhibitory control. Upon infection, neurons secrete mitogenic signals that stimulate SCs to proliferate vigorously (Wen *et al.*, 1994). However, the molecular mechanisms involved are not fully understood and it is thought that the benefits of SC proliferation are primarily in preventing trans-neuronal spread (Elson *et al.*, 2003).

PNS neurons can themselves prevent their own destruction due to certain physiological features they possess. Firstly, they are able to down-regulate the formation of MHC class I antigen and thus impede recognition by $CD8^+$ T cells (Wong *et al.*, 1984). In normal conditions neurons do not express MHC class I (Oldstone, 1991). Secondly, due to their differentiation they may lack transcription factors required for efficient IE viral gene transcription (Valyi-Nagy *et al.*, 1991) or they possess repressors preventing it in the first place (Kemp *et al.*, 1990).

7.1.2.1 Immune evasion strategies by HSV1

Even though incapable of antigenic variation, HSV1 has evolved mechanisms that allow evasion of detection from host immune surveillance. These mechanisms target both the ability of the host to initiate cytotoxic and antibody/complement mediated responses as part of the innate immune response as well as inhibition of mounting an adaptive immune response. HSV1 can interfere with antigen presentation to $CD4^+$/$CD8^+$ T cells. It achieves this by preventing the expression of MHC class II protein, or by inhibiting the binding of antigen to MHC class I protein. Blocking of viral antigen presentation to MHC class I specific $CD8^+$ CTLs is prominent during lytic infection and not in latency (Pereira *et al.*, 1994). Experimental evidence supports the fact that ICP47, even though not an essential protein (Mavromara-Nazos *et al.*, 1986), plays a crucial role in this mechanism (Goldsmith *et al.*, 1998). Normally, the viral antigen is loaded onto empty MHC class I proteins in the endoplasmic reticulum (ER). Transporter-associated with antigen processing (TAP) proteins (Bauer and Tampe, 2002), mediate transport of the viral antigen to the ER. It is thought that ICP47 binds to the cytosolic portion of the TAP transporter, thus preventing its translocation into the ER and the presentation of MHC class I - viral antigen complex to $CD8^+$ T cells (Fruh *et al.*, 1995). MHC Class I molecules remain unloaded in the ER (Bauer and Tampe, 2002). Interestingly, ICP47 expressing cells show a similar MHC class I instability to that of TAP deficient in cells (Fruh *et al.*, 1995). In addition, TAP has been detected using ICP47 antibodies, further elucidating the direct interaction of the two proteins (Goldsmith *et al.*, 1998). It is worth noting that there is some evidence to suggest that the strength and nature of that interaction is species specific. HSV1 ICP47 was found to inhibit human TAP but ICP47 concentrations of 50 to 100 times more were necessary to efficiently block the murine version of TAP (Tomazin *et al.*, 1998). HSV1 has also been reported to interfere with the function and maturation of dendritic cells during the initial stages of infection (Sprecher and Becker, 1987). Infected dendritic cells, even though non-permissive to virus growth themselves, are prevented from undergoing maturation and they are therefore incapable of presenting viral antigens to either $CD4^+$ or $CD8^+$ T cells (Pollara *et al.*, 2003; Sprecher and Becker, 1987). This inefficiency allows HSV1 more time to spread (Mikloska

and Cunningham, 2001). A recent study suggests that this block of dendritic cell maturation is a consequence of the HSV1 Vhs protein. In fact, deleting vhs allows the activation of dendritic cells and efficient presentation of antigens to T cells (Samady *et al.*, 2003).

MHC class II processing pathway is also thought to be targeted by HSV1 (Lewandowski *et al.*, 1993). As previously reported, generation of Th1/Th2 $CD4^+$ effector cells and subsequent activation of plasma cells, requires interaction with viral peptides loaded onto MHCII αβ heterodimers. Peptide loading of MHCII heterodimers takes place in endosomal vesicles. Normally, MHCII molecules are found associated with the invariant chain peptide (Castellino and Germain, 1995) that blocks the binding groove of MHCII to ensure correct loading of antigen while preventing random association with self peptides (Roche and Cresswell, 1991). Later in the maturation process, human leukaemia antigens-DM (HLA-DM) heterodimers remove the invariant chain (Denzin and Cresswell, 1995) and further processes bound peptides for stronger structural binding to MHCII (Sanderson *et al.*, 1994). Several HSV1 antigens are thought to have a negative impact on the molecular processing of MHCII. Firstly, HSV1 infection strongly down-regulates the expression of the invariant chain which in turn prevents the formation of DR-peptide complexes. Secondly, the viral envelope protein gB can bind to DR (Sievers *et al.*, 2002) and prevent it from binding to the invariant chain. Thirdly, gB-associated DR and DM heterodimers are exported from the ER but are primarily localised in sub-cellular vesicles which are prevented from being expressed on the surface of the infected cell (Neumann *et al.*, 2003).

Apart from interfering with MHCI/MHCII processing and antigen presentation, HSV1 is also capable of evading complement attack *in vivo*. It accomplishes this by two mechanisms, either by mimicking complement regulators or by preventing complement binding to antibody-antigen complexes (Favoreel *et al.*, 2003). Viral glycoprotein gE synergises with gI (Dubin *et al.*, 1991; Johnson and Feenstra, 1987) and binds host IgG (Para *et al.*, 1982) via its immunonoglobulin binding domain which demonstrates a high similarity to mammalian Fc receptors (Dubin *et al.*, 1990; Dubin *et al.*, 1994). The gE-gI functions as an Fc receptor and allows the virus to avoid complement-mediated lysis via a process described

as anibody biopolar bridging (Frank and Friedman, 1989). This consists of IgG binding with its hypervariable Fab region to a viral envelope or cell surface protein and with its Fc region to the HSV Fc receptor gE-gI which in turn interferes with C1q binding and antibody-dependent cytotoxicity (Dubin *et al.*, 1991). This protects the virus from complement and antibody dependent neutralisation *in vitro* and *in vivo* (Frank and Friedman, 1989; Lubinski *et al.*, 2002; Nagashunmugam *et al.*, 1998). Indeed, mutant strains of HSV1 in which gE binding to IgG is defective, display a 40 to 100 fold increase in susceptibility to complement neutralisation (Nagashunmugam *et al.*, 1998).

Glycoprotein gC is a non-essential glycoprotein, present on the viral envelope and on the surface of infected cells, known to play a role in viral attachment, release and virulence (Herold *et al.*, 1991). gC can act as an immunoevasin by binding to complement components and its activation products C3, iC3b and C3c and it can accelerate the decay of the C3 convertase in the alternative pathway and *in vivo*. Viral gC also interferes with C5 and properdin from binding to C3b (Fries *et al.*, 1986; Kostavasili *et al.*, 1997). Properdin's role is to increase the half-life of the alternative C3 convertase whereas C5 is an important complement component by participating in the formation of the terminal, membrane associated complex (MAC) (Favoreel *et al.*, 2003). Both proteins require binding to C3b in order to perform these functions. Inhibition of properdin is carried out by the transmembrane segment of gC while inhibition of C5 binding to C3b is the result of steric hindrance, preventing the C5 binding site from accessing the C3b binding site (Kostavasili *et al.*, 1997).

In addition, ICP34.5 has been associated with a mechanism that can counteract the virally induced pro-inflammatory interferon response (Samuel, 2001). HSV1 can target both Type I (IFNα and IFNβ) and Type II (IFNγ) interferons which are involved in the primary or productive phases of viral infection (Male *et al.*, 1996). Type I proteins IFNα and IFNβ are secreted by all virally infected nucleated cells especially fibroblasts, macrophages and dendritic cells. On the other hand, IFNγ is specific to cells of the immune system such as NK cells, $CD4^+$ Th1 and γ/δ $CD8^+$ T cells (Bach *et al.*, 1997). Even though the actions of Type I or II interferons are initiated via different receptors, they converge on the

JAK/STAT signalling pathway ultimately resulting in the up-regulated expression of specific interferon-responsive genes. However, the expression of interferon-responsive genes can also be initiated by virus infection directly, and in cases such as that of paramyxovirus infections, the signalling mechanisms differ from those used by the cytokines themselves in that the JAK/STAT signalling pathway is bypassed (Guo *et al.*, 2000; Marie *et al.*, 1998; Wathelet *et al.*, 1998).

The spectra of cellular genes induced by interferons and by viral infection are similar but not identical, with some genes responding to either or both inducers (Wathelet *et al.*, 1992). One such interferon-responsive gene is the *PKR* gene. PKR is ubiquitously expressed in mammalian tissues, is maintained in an inactive form and belongs to the eukaryotic initiation factor-2 subunit α (eIF-2α) family of protein kinases (Lu *et al.*, 1999). Upon a positive signal, induced by either IFN or the presence of double stranded RNA, PKR undergoes auto-phosphorylation and a conformational alteration that allows dimerisation into the now active PKR (Gale, Jr. and Katze, 1998). PKR then phosphorylates and inactivates eIF-2α which as a result leads to a total shut down of translation within the host cell, be it host or viral (Gale, Jr. and Katze, 1998). This ultimately leads to the death of the host cell by apoptosis (Kaufman, 1999). A study in which trigeminal ganglia cultures were transduced to express IFNβ by means of an adenoviral vector, demonstrated that PKR is up regulated in response to a concomitant HSV1 infection (Al khatib *et al.*, 2003; Carr *et al.*, 2003). HSV1 has evolved a specific mechanism in order to avoid this response. The carboxy-terminal domain of the ICP34.5 binds to protein phosphatase 1-α (PP1α) and directs PP1α to reverse the phosphorylation of eIF-2α mediated by PKR (He *et al.*, 1997; He *et al.*, 1998; Pasieka *et al.*, 2006), thus allowing translation to resume. It must however be pointed out that this effect has only been demonstrated *in vitro*. Moreover, an ICP34.5 mutant is capable of exhibiting wild-type replication levels in mice lacking the PKR gene (Leib *et al.*, 2000). The authors of this study suggest that this restoration of virulence is specific to ICP34.5 since a virus deleted for viral thymidine kinase (TK) failed to reach wild type replication levels in the same paradigm. Recently, more HSV1 proteins have been implicated with PKR control.

Another viral protein US11, an RNA binding protein, can compensate for the absence of ICP34.5 and it was shown to directly associate with PKR early in infection thus decreasing its activation both *in vitro* (Cassady *et al.*, 1998) and *in vivo* (Ward *et al.*, 2003). Studies directed at evaluating the responsiveness of individual HSV1 genes to interferons demonstrated that ICP0 mutants are hypersensitive to IFNα *in vitro* compared to the less sensitive VP16, UL13, vhs or ICP22 mutants (Mossman *et al.*, 2000). When ICP0 mutants were grown on complementary cell lines they displayed normal sensitivity to IFNα. This might suggest that ICP0 interferes with IFNα responses and may act as a regulator on sensitivity. Whether this response is separate or part of the ICP34.5 response is currently not clear (Mossman and Smiley, 2002). A recent report suggests that the ICP0 functions as an antagonist to the functions of not only IFNα but also IFNβ and IFNγ which would explain why wild type HSV1 is apparently highly resistant to the hostile state induced by the activation of the IFNα, IFNβ and IFNγ receptors (Harle *et al.*, 2002). This is in sharp contrast to the ICP0$^-$ versions of the virus, that appear to be hypersensitive to the activation of these receptors *in vitro* (Mossman and Smiley, 2002) while being able to replicate with improved efficiency in knockout mice that lack ICP0 and ICP34.5 (Leib *et al.*, 1999). IFNα, IFNβ and IFNγ receptors activate their effectors via the activation of the Stat-1 transcription factor which ICP0 appears to directly antagonise (Halford *et al.*, 2006). In the experiments by Halford and colleagues (2006) it was demonstrated that in mice lacking Stat-1, infection with an ICP0$^-$ mutant was lethal. The authors suggest that when ICP0 is present, it is able to overcome the HSV1 genome silencing that occurs in the presence of stat-1, which is in turn activated by all three INFs. These authors go on to suggest that ICP0 is essentially a switch that decides whether the virus will enter replication, i.e. whether it is able to overcome the stat-1 induced silencing, or enter latency instead (Halford *et al.*, 2006).

HSV1 can also down-regulate the production of a group of pro-inflammatory cytokines, such as Interleukin 6 (IL6) (Mogensen *et al.*, 2004). This ability is attributed to the viral IE genes ICP4 and ICP27, which are expressed in a VP16-dependent manner during primary HSV infection. Interestingly and from the

same study it was elucidated that after infection with an VP16 *in1814* mutant, IL6 mRNA demonstrates a significantly longer half-life compared to that observed after infection with the wild type virus (Mogensen *et al.*, 2004). Based on these results, Mogensen and colleagues (2004) proposed that HSV is able to suppress expression of pro-inflammatory cytokines by decreasing the stability of mRNAs, thereby potentially impeding the antiviral host responses to infection. The HSV1 vector used for the regenerative studies in the CNS described in this thesis, has both ICP34.5 and VP16 disabled. As discussed in Chapter 3, it is possible that the lack of transgene expression in cortical neurons is likely to be due to the silencing of the exogenous promoter that does not appear to occur when these genes are present, as in the case of less disabled vectors. However, it is also likely that the long-term transgene expression in highly disabled constructs may be partly due to an inability to evade the host immune response.

7.1.2.2 HSV1 Viral Vectors ~ Anti-Apoptotic Signals

Apart from immune detection, the virus can also suppress apoptotic signals within the infected cell. Two viral proteins have been suggested for their involvement in this mechanism: ICP4 and ICP34.5. 40-50% of Vero cells infected with $ICP4^-$ mutants show apoptotic signs, i.e. DNA fragmentation, obliteration of the nuclear membrane and vacuolisation of the cytoplasm (Leopardi and Roizman, 1996). On the contrary, cells infected with an $ICP4^+$ virus that was unable to express any of the β or γ genes, had the characteristics of an infected cell but no apoptotic signs. The mechanism of action of the proposed anti-apoptotic function of ICP4 remains largely unknown. In addition to ICP4, the ability of ICP34.5 to prevent apoptosis has also been examined. Initially it was noted that γ34.5 deletion mutants induced apoptosis in human neuroblastoma cells, via the phosphorylation of the eukaryotic translation initiation factor eIF-2a (Chou and Roizman, 1992), hence leading to the premature shut-off of host protein synthesis. In another line of experimentation, the ability of an ICP34.5 deleted HSV1 viral vector to safely deliver transgenes to the brain was found to be limited (McMenamin *et al.*, 1998). The animals, injected in the brain, suffered severe weight loss and experienced a powerful immune response. Furthermore, $\gamma34.5^-$ mutants where compared to wild type HSV1 for their ability to suppress

DNA fragmentation in Hep-2 carcinoma cells (Koyama and Miwa, 1997). Hep-2 cells were stimulated to undergo apoptosis by sorbitol treatment, prior to infection. Surprisingly, the deleted virus was as effective as wild type HSV1 in suppressing DNA fragmentation. The authors of this study reasoned that more viral proteins are responsible for the inhibition of apoptosis in infected Hep-2 cells.

Another theory concerning the anti-apoptotic abilities of HSV1 regards the fact that the LAT transcript in itself has the ability to protect infected neurons from programmed cell death, as an innate mechanism to make latent infections more persistent (Perng *et al.*, 2000; Thompson and Sawtell, 2001). Replacing LAT with an antiapoptosis gene restores the wild-type reactivation phenotype in a LAT null mutant of HSV1 (Perng *et al.*, 2002). In addition, a plasmid expressing a fragment of LAT between nucleotides 301-2659*nt* can efficiently block apoptosis in transient transfection assays, further demonstrating its powerful anti-apoptotic activity (Perng *et al.*, 2000).

7.1.3 Interleukin 10 & Immunosuppression

Interleukin 10 (IL10) has been identified as a natural anti-inflammatory cytokine, produced by $CD4^+$ Th-2 cells, activated B cells and macrophages (Howard and O'Garra, 1992). It inhibits activation and proliferation of T-cells indirectly, by reducing the antigen-presentation capacity of monocytes. This inhibition is achieved by reducing the expression of co-stimulatory molecules, such as CD80 or CD54 (Chang *et al.*, 1994), and MHC class II presentation by monocytes (Chang *et al.*, 1994). IL10, has also been shown to ameliorate the effects of septic shock that can be induced by TNFβ (Griffith *et al.*, 1995). In the absence of APCs, IL10 can suppress IL2 and TNF-α secretion by $CD4^+$ Th1 cells and can induce long lasting antigen specific anergy (Groux *et al.*, 1996; Groux *et al.*, 1998). Interestingly, repetitive antigen activation of $CD4^+$ cells in the presence of IL10 results in the differentiation of a new subset of regulatory cells termed T-regulatory cells-1 (Tr1). These cells have a poor proliferative response, do not produce IL2 and advantageously, produce high levels of IL10 (Groux *et al.*, 1997).

IL10 has been further shown to inhibit IFNα production by monocytes and natural IFNα producing cells (NIPCs) in response to viral infections (Cederblad and Alm, 1991). HSV1 infection results in IFNα up-regulation by NIPCs, which in the CNS leads to an increase in expression of MHC class II by astrocytes (Payvandi *et al.*, 1998). In addition, IL10 inhibits the production of nitric oxide and IL12 production by activated microglia, suggesting an important role for this cytokine as a negative regulator of a CNS immune response (Lodge and Sriram, 1996). More evidence for the IL10 regulatory function in suppressing a CNS immune response comes from studies where BALB/c and SJL strains of mice were compared for their susceptibility to Sindbis virus infection (SV) (Rowell and Griffin, 1999). SV causes acute nonfatal encephalomyelitis in both strains of mice. SJL mice are susceptible to autoimmune inflammatory diseases of the CNS, while BALB/c mice are relatively resistant. Even though clearance of virus was similar in both strains, SJL mice developed a more intense inflammatory response in the brain and spinal cord than the BALB/c mice and inflammation persisted for several weeks. Analysis of lymphocytes present in brains isolated early after infection, showed an absence of activated NK cells in SJL mice. In contrast to its anti-inflammatory and immunosuppressive properties, IL10 can have immuno-stimulatory and anti-apoptotic effects in both B and T cells. In addition, it has been shown to augment the proliferation of spleenic activated B cells into antibody secreting cells. In a mouse transgenic model, IL10 enhances the accumulation of $CD8^+$ cells in the pancreas, leading to diabetes (Groux *et al.*, 1999). Additionally, IL10 has been shown to rescue T-cells from apoptosis when IL2 is not present. Injection of high doses of IL10 in mice with graft vs. host disease, resulted in exacerbation of the disease (Krenger *et al.*, 1994). The benefits of IL10 have been demonstrated in transplantation and various disease models. For example, local production of IL10, mediated by adenovirus vectors, by canine islets, improves early islet survival in rat recipients (Deng *et al.*, 1997). In addition, adenoviral transfer of the Epstein-Barr derived homologue of the IL10 gene (the BCRF1 protein) to mouse paws, suppressed the development of collagen-induced arthritis (Whalen *et al.*, 1999). It has been postulated that loss of vector-mediated transgene expression is the result of immune clearance of the infected cells rather than a cell specific down regulation of transgene expression

(Palmer *et al.*, 2000). The ability of IL10 to promote immuno-suppression establishes this interleukin as an attractive molecule that could potentially allow longer transgene expressions when co-expressed by the vector, allowing it to evade immune surveillance mechanisms.

7.1.4 Rationale

The experiments described in this study aimed to test the hypothesis that if immune system clearance is the cause of the short-lived HSV1.pR20.9/UL43-mediated transgene expression noted in DRG neurons, then suppressing the host's immune response may lead to transgene expression that can persist for longer time points in these neurons. This could be measured in terms of longer transgene expression in target cells.

To elucidate any potential immune system involvement, GFP/LacZ transgene expression was compared between BALB/c and the immunocompromised Severe Combined Immunodeficiency Disease (SCID) mice at different time points using the backbone vector HSV1.pR20.9/UL43. SCID mice suffer from an abnormality that selectively kills lymphocytes. As they have neither T nor B-lymphocytes they are unable to generate an immune response and can therefore provide important data regarding the involvement of the immune system in shutting down transgene expression. If there is no immune system involvement then there may be no differences between BALB/c mice and the immunocompromised SCID mice in terms of the longevity of transgene expression. In order to suppress the immune system response in host animals, a new vector was constructed (termed HSV1.pR20.9mIL10/UL43). This vector co-expresses the LacZ marker gene along with the potent anti-inflammatory cytokine IL10. In the experiments described here, transgene expression in the immune-competent BALB/c DRG neurons achieved with the IL10 expressing vector is evaluated at longer time points and compared to that achieved with vector HSV1.pR20.9/UL43.

7.2 Methods

7.2.1 Construction and characterisation of HSV1.pR20.9mIL10/UL43

Vector HSV1.pR20.9/UL43 (1764/pR20.9UL43) has been well characterised for its ability to simultaneously deliver two transgenes (GFP and LacZ) into adult mice DRGs (Lilley *et al.*, 2001b; Palmer *et al.*, 2000) and presented the ideal candidate for the delivery of mouse IL10. This vector is based on the wild type HSV1 strain *17+* (Brown *et al.*, 1973), is deleted for both copies of the neurovirulence factor ICP34.5 (MacLean *et al.*, 1991) and is inactivated for VP16 via the *in1814* mutation (Ace *et al.*, 1989). The lack of ICP34.5 hinders the primary envelopment of the virus and thus prevents the virus from infecting other cells and cross trans-synaptically. (Bjerke *et al.*, 2003). The bicistronic promoter construct pR20.9 is inserted into the UL43 gene at the unique *NsiI* site (*nt 94,911*). In order to create a vector based on this backbone, capable of delivering biologically active mIL10, it was first necessary to sub-clone the cDNA into the pR20.9UL43 expression cassette, substituting the GFP marker gene. As pR20.9 is a bicistronic cassette, it allows monitoring of how LacZ expression is affected when IL10 is co-expressed in adult BALB/c or SCID mouse DRGs.

7.2.1.1 Cloning of mIL10 into pGEM2/pR20.9UL43

cDNA encoding for mIL10 (Kim *et al.*, 1992) (GeneBank accession number M84340) was a gift from Oxford University (Dept. of Anatomy). mIL10 cDNA (≈600bp) was removed by digesting first with *Not*I, blunted using T4 DNA Polymerase and finally digested with *Hind*III. The fragment was then sub cloned into the pR20.9 shuttle plasmid flanked by UL43 sequences. The pR20.9 expression cassette is flanked by homologous HSV1 UL43 sequences that enable it to be inserted by homologous recombination into the UL43 region located in the unique long region of the viral genome (Palmer *et al.*, 2000). The reporter gene GFP of the vector backbone pGEM2/pR20.9UL43, was removed by enzymatic digest using *Xho*I followed by blunt ending with T4 DNA polymerase and restriction digest with *Hind*III. After ligation, mIL10 containing clones were confirmed by screening multiple restriction digests for the correct restriction profile. The new plasmid generated was termed pGEM2/pR20.9mIL10/UL43.

7.2.1.2 Production of HSV1.pR20.9mIL10/UL43 vector

Purified HSV1.pR20.9/UL43 (1764/pR20.9/UL43) viral DNA was co-transfected with pGEM2/pR20.9mIL10/UL43 plasmid DNA. Recombinant plaques were detected by their white phenotype (no GFP expression), indicative of a correct recombination event and insertion of the pGEM2/pR20.9mIL10/UL43 expression cassette into the UL43 gene of the 1764 backbone, at the unique *Nsi*I site (*nt 94,911*) (Palmer *et al.*, 2000). Plaque purification was carried out until all plaques had the recombinant, non-GFP expressing but LacZ expressing phenotype. The new vector was termed HSV1.pR20.9mIL10/UL43 (1764/pR20.9mIL10/UL43) (Figure 7.4.1-a). Correct insertion of the mIL10 gene was confirmed by Southern blotting on all purified recombinant plaques, originating from separate recombination events. The recombinant vector was propagated by standard cell culture techniques on BHK cells and viral stocks were produced as outlined in Chapter 2.

7.2.1.3 Southern blotting on HSV1.pR20.9mIL10/UL43 plaques

Each purified recombinant HSV1.pR20.9mIL10/UL43 viral plaque was used to infect $1x10^6$ BHK cells in a 36 mm well tissue culture plate. Viral DNA from each plaque was extracted as outlined in Chapter2, digested with *Hind*III/*Nsi*I and run on a 2% agarose gel. HSV1.pR20.9/UL43 viral DNA digested by *Hind*III/*Nsi*I (releasing a 600bp fragment corresponding to GFP) was used as a negative control while a *Hind*III/*Not*I digested plasmid pXCXR-mIL10 was used as a positive control. To produce the mIL10 specific probe, the pXCXR-mIL10 plasmid was digested with *Hind*III/*Not*I and the mIL10 600bp fragment produced was purified and labelled with αP^{32}-dCTP.

7.2.1.4 Isolation & concentration of secreted mIL10 from cultured BHKs

$1x10^6$ BHK cells in a 36 mm well were infected at an MOI of 1. Two days later the infected monolayer was washed twice with 0.1M PB, 500 µl of DMEM were aliquoted in each well and the cells were returned to a 37°C, 5% CO_2 incubator. At 2, 4 and 8 hour time points, the DMEM was collected and centrifuged at 3,000G for 5 minutes to remove any cells present. The supernatant was then

transferred onto a 500 µl Microcon column with a 10kDa molecular weight cut-off (G-10000, Green). The column was then spun at 11,500G for 25 minutes at 4°C. The total volume of the concentrated sample was 20-25 µl, which represents the amount of mIL10 secreted from $1x10^6$ BHK cells infected with HSV1.pR20.9mIL10/UL43.

7.2.1.5 Western blotting

Secreted mIL10 from $1x10^6$ BHK cells previously infected with HSV1.pR20.9mIL10/UL43 at an MOI of 1 was obtained as outlined above. Expression was analysed at 2, 4 and 8-hour time points. An equal volume of protein sample buffer [50mM Tris-HCl pH 8.0, 2% (w/v) SDS, 6% (v/v) glycerol and 0.005% (w/v) bromophenol blue] was added to each sample. Samples were placed on ice immediately and then denatured by heating to 95°C for 4 minutes. They were then cooled on ice for 2 minutes and either loaded onto a 15% SDS-polyacrylamide gel or stored at -20°C. SDS-PAGE and Western blotting were performed as described in Chapter 2. All the gels run were normalised for total protein content and all nitrocellulose membranes were stained with 1% (w/v) Panceau stain to ensure equal loading.

7.2.2 Animal Surgery - Foot-pad inoculations in adult mice

Three-week-old female BALB/c or SCID mice were inoculated subcutaneously into the plantar surface of the right hind paw with either HSV1.pR20.9/UL43 or HSV1.pR20.9mIL10/UL43 vectors (≈1-2$x10^6$ pfu) at a volume of 20-25 µl. In the case of BALB/c mice it was possible for them to be sedated under brief volatile anaesthesia with (3% Isofluothane in oxygen) throughout the procedure. In the case of SCID mice this was not possible and injections had to be carried out in a sterile hood without anaesthesia. At the predetermined time points (2 weeks and 2 months), mice were sacrificed and DRGs from the lumbar spine (L1 to L6) and ipsilateral to the inoculated side were extracted, post-fixed for 1 hour in 4% (w/v) PFA and examined first for the expression of GFP and then processed with X-gal stain. None of the controlateral DRGs in any of the animals used in this experiment were found to be infected with the vector.

7.3 Results

7.3.1 Characterisation of vector HSV1.pR20.9mIL10/UL43

The recombinant HSV1.pR20.9mIL10/UL43 vector was produced by the co-transfection of plasmid pGEM2/pR20.9mIL10/UL43 with purified HSV1.pR20.9/UL43 viral DNA. As the UL43 gene exists in one copy, located in the unique long region of the HSV1 genome, the viral plaques that displayed a $GFP^-/LacZ^+$ phenotype were selected. Purification of the six HSV1.pR20.9mIL10/UL43 plaques initially selected was achieved in nine rounds of purification.

In order to confirm that the $GFP^-/LacZ^+$ phenotype observed was due to the correct recombination event and not due to a random mutation of the GFP gene, all purified plaques were subjected to Southern blot analysis. Each of the six different constructs purified was used to infect $1x10^6$ BHK cells and the presence of the mIL10 cDNA was confirmed using a mIL10-specific αP^{32}-dCTP radiolabelled probe, originating from the pXCXR-mIL10 plasmid. As can be seen in Figure 7.3.1-b (panel **A**), out of the six different constructs purified, only two were found to be positive for the presence of mIL10 cDNA. Even though a thorough investigation into the reasons for this was not thought necessary, two possibilities arise. Firstly, it is possible that a random mutation of the GFP gene prevented the phenotype from presenting while the recombination event was in itself unsuccessful. Secondly, it is possible that a recombination event between the LAP1 and LATP2 regions within the pR20.9 cassette and the endogenous LAT region resulted in the loss of either the GFP or mIL10 genes. In both these cases, the resulting plaque phenotype would be white and blue, even though the recombination event was not due to the successful insertion of mIL10. The latter recombination event has been noted before in our laboratory when using this type of vector and it is more frequent when a viral stock has been extensively propagated.

Successful expression of mIL10 was confirmed by Western blot analysis of supernatants obtained from HSV1.pR20.9mIL10/UL43 transduced, BHK cells.

$1x10^6$ cells were infected with an MOI of 1 and supernatants were collected after 2, 4 and 8 hours. Supernatants were screened for the presence of mIL10 using a Goat polyclonal anti-mouse IL10 antibody (Santa Cruz, SC-1718) and compared to supernatants obtained from HSV1.pR20.9/UL43 transduced cells or recombinant murine IL10. As it can be seen in Figure 7.3.1-b (panel **B**), vector encoded mIL10 expression is significant at 2 hours, while the two later time points confirm that expression is continuous with increasing amounts of mIL10 accumulating in the tissue culture supernatants.

Murine IL10 is composed of 174 amino acids (Kim *et al.*, 1992; Moore *et al.*, 1990) which determine the purely α-helical, secondary structure of the protein. The mature protein has a molecular weight of approximately 18 kDa and when biologically active, it forms non-covalent 30 kDa homodimers. This tertiary form is possible due to two disulfide bonds (Windsor *et al.*, 1993) forming between monomers. Whether the protein remains a monomer (18 kDa) or associates into a dimer (30 kDa) depends on the pH of the solution and the presence of a reducing agent such as mercaptoethanol. mIL10 predominates as a homodimer at neutral pH, migrating as a single band to 30 kDa while at acidic pH only the 18 kDa monomer is present (Syto *et al.*, 1998). Based on this observation and on the fact that, at least for the human analogue of the protein, it is the dimer that is biologically active (Syto *et al.*, 1998), it was decided to analyse the HSV1.pR20.9mIL10/UL43 delivered mIL10 under non-reducing conditions (pH ≈5.2). As it is shown in Figure 7.3.1-b (panel **B**), it was possible to see both forms of the protein in the absence of a reducing agent. Even though the pH of the sample and that of the SDS-Page gel are alkaline thus favouring the dimeric structure, this association is disturbed by the length and temperature of incubation which in turn favours the monomer. This is not observed for the recombinant mouse IL10 (R&D Systems, 474-MR) as it is formulated with a cross-linking agent and is thus resistant to alterations of pH and temperature. In addition, there appears to be no cross reactivity of the antibody with any other viral or cell proteins.

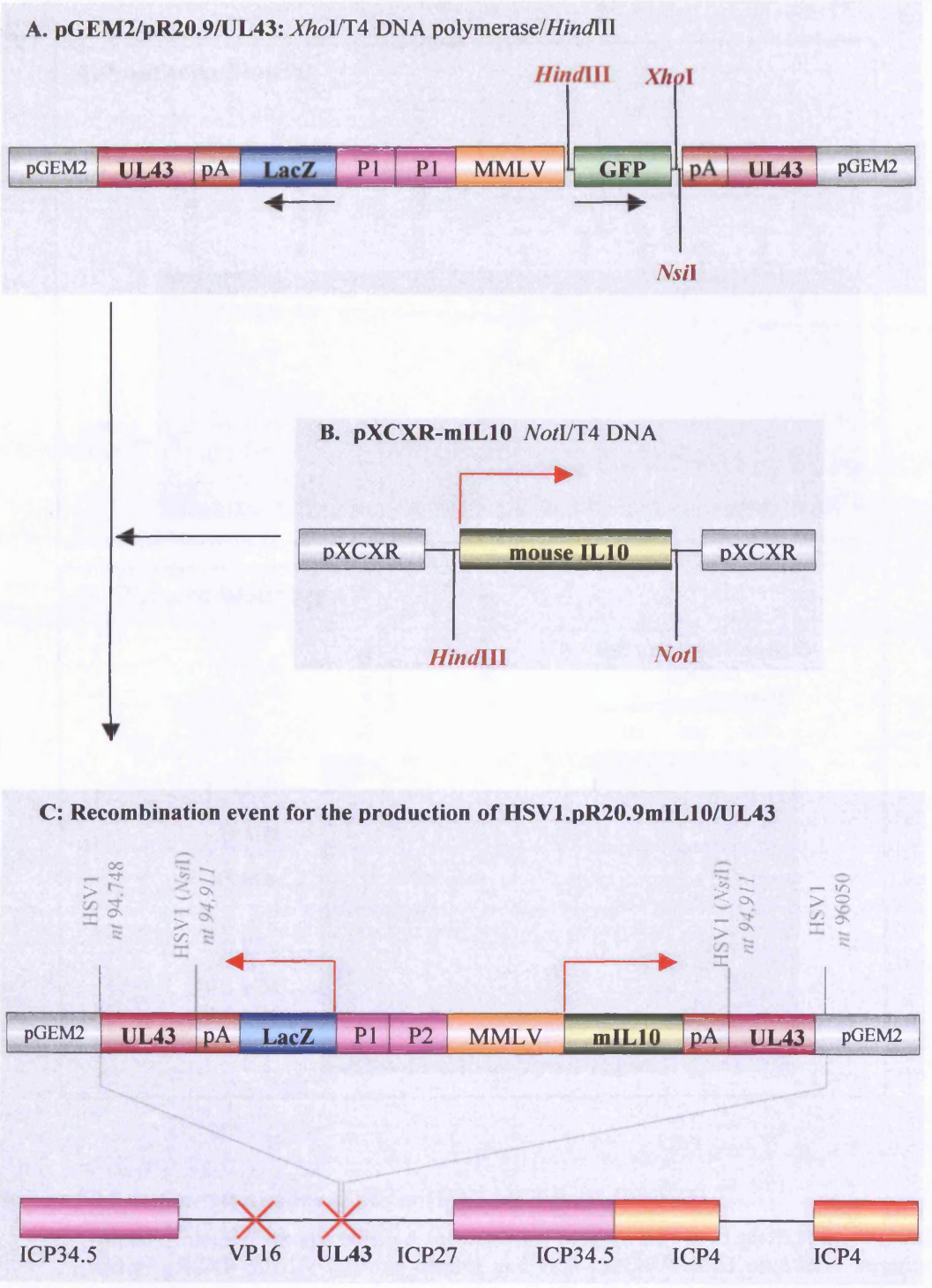


Figure 7.3.1-a: Construction of HSV1.pR20.9mIL20/UL43.

The modified plasmid pGEM2/pP20.9mIL10/UL43 was recombined into the endogenous UL43 gene.

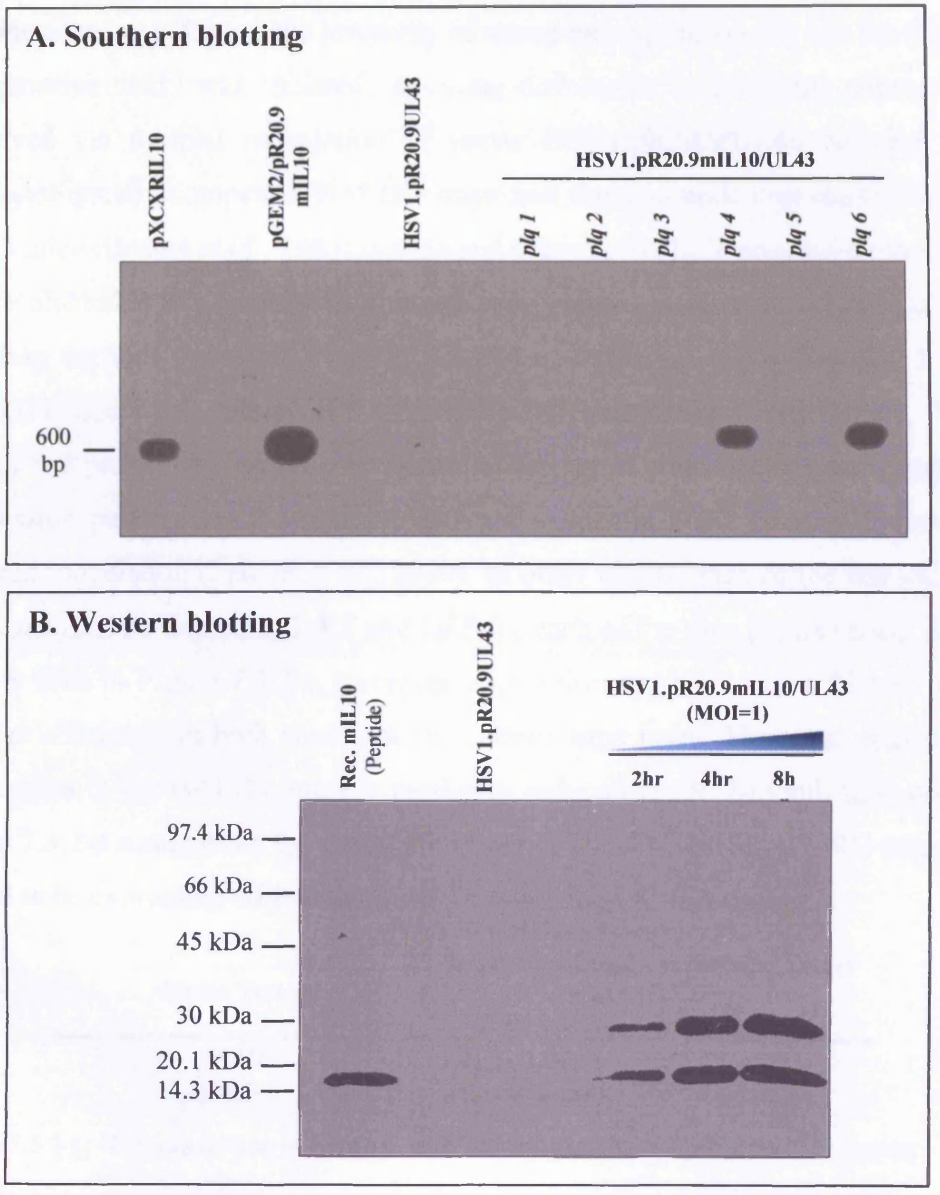


Figure 7.3.1-b: Characterisation of vector HSV1.pR20.9mIL10/UL43.

A. Southern blotting on six purified recombinant plaques of HSV1.pR20.9mIL10/UL43 vector. pXCXR-mIL10: positive control and HSV1.pR20.9/UL43 used as a negative control. The only band recognised from the mIL10 probe corresponds to the mIL10 gene (600bp).

B. Western blot probed with Goat polyclonal anti-mouse IL10 (Santa Cruz, SC-1783). Recombinant mouse IL10 (blocking peptide, Santa Cruz, SC-1783P) and loaded at 50 μg/ml to act as a positive control. The supernatant obtained from BHK cells infected with HSV1.pR20.9/UL43 did not produce any bands that cross-reacted with the primary antibody used endorsing its specificity to murine IL10.

7.3.2 Involvement of the immune system on transgene expression

This experiment was designed in order to demonstrate whether the host's immune response has an effect on the longevity of transgene expression. To test for this, a comparative study was initiated, assessing differences in transgene expression achieved via footpad inoculation of vector HSV1.pR20.9/UL43 between the immunologically-competent BALB/c mice and the immunocompromised strain SCID mice (Bosma *et al.*, 1983; Bosma and Carroll, 1991). Transgene expression was evaluated at the 2-week and 2 month time points. As already discussed in the previous section, vector HSV1.pR20.9/UL43 co-expresses two transgenes: LacZ and GFP under the control of LAP1 and MMLV promoters respectively. This vector had previously been shown in our laboratory to consistently have identical expression patterns for either of the two transgenes in DRG neurons following footpad inoculation (Palmer *et al.*, 2000). In other words, each of the transduced DRG neurons co-expresses LacZ and GFP for each of the time points tested. As it can be seen in Figure 7.3.2-a, transgene expression appears to be achieved with similar efficiency in both strains at the 2-week time point. However, transgene expression in the BALB/c mice is markedly reduced at the 2-month time point. Table 7.3.2-a summarises the average number of BALB/c or SCID DRG neurons found to be expressing GFP at the 2 weeks and 2 months time point.

Strain Tested	Number of LacZ - expressing DRGs (Mean ±SD)	
	2 weeks	2 months
SCID	205 ± 81 (n=5)	118 ± 43 (n=5)
BALB/c	213 ± 60 (n=5)	7 ± 4 (n=5)

Table 7.3.2-a: GFP expression in BALB/c & SCID mice via HSV1.pR20.9/UL43 vector.

The above results demonstrate a difference between BALB/c and SCID mice. It appears that at 2 months post injection expression of LacZ persists in 118±43 DRG neurons of SCID mice and in 7±4 BALB/c mice. Even though at the 2-months time point transgene expression has deteriorated in both strains, there are still more transduced DRG neurons in SCID mice (Table 7.3.2-a). This points to a possible immune system involvement to the fading transgene expression via the HSV1.pR20.9/UL43 vector. Immunomodulation by means of mIL10 should therefore be a suitable approach for extending the period of transgene expression.

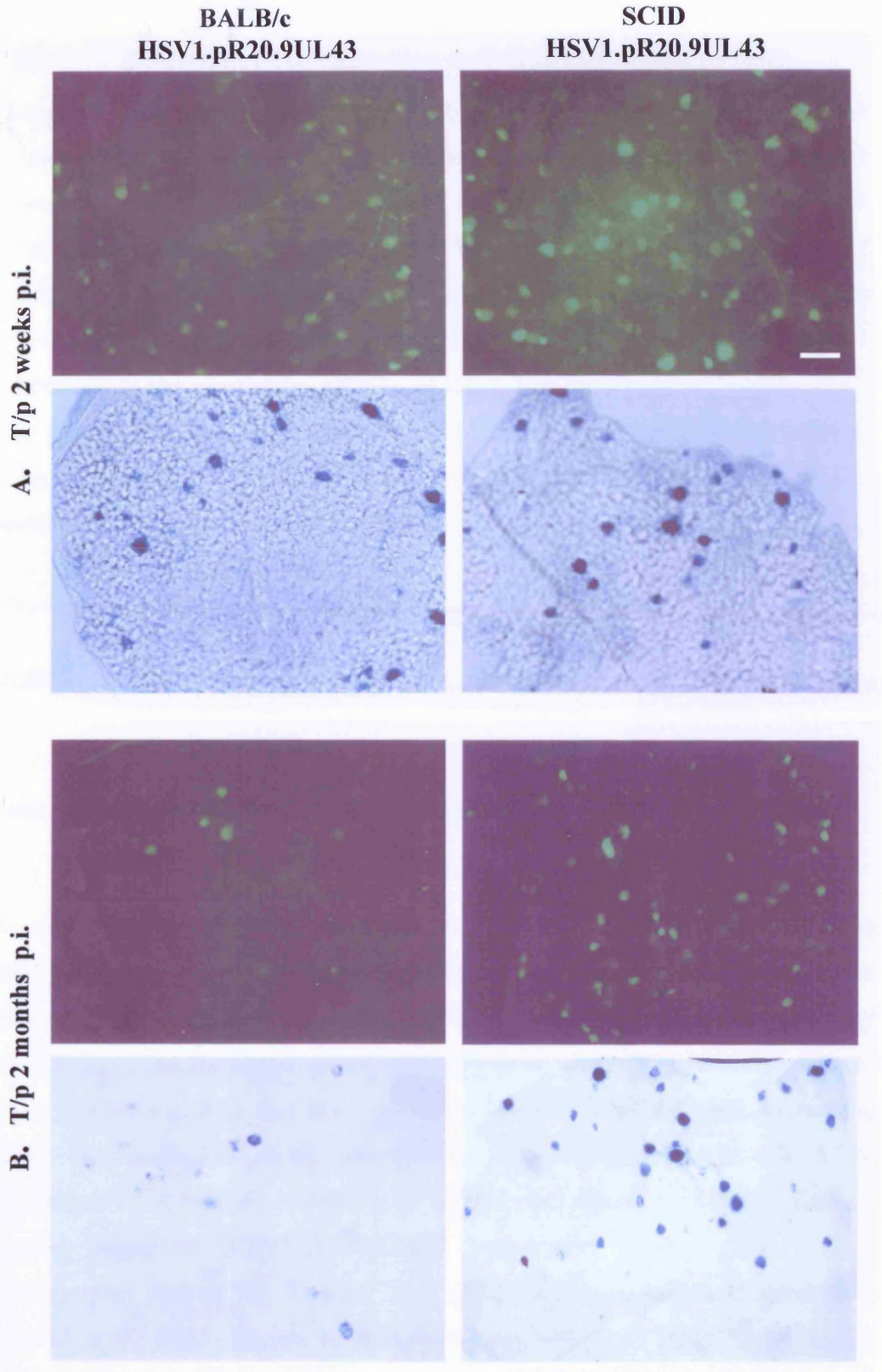


Figure 7.3.2-a: HSV1.pR20.9/UL43 LacZ & GFP expression in BALB/c & SCID mice.

GFP vector transduced DRG neurons (green) and LacZ vector transduced DRG neurons (blue) in wholemounts of DRG, 2 weeks (**A**) and 2 months (**B**) after injection into the footpad of BALB/c mice (left) and SCID mice (right). Note the larger number of transduced neurons in SCID compared to BALB/c mice at the two month time point. Scale bar: 150µm, applies to all figures.

7.3.3 Effect of IL10 co-expression on BALB/c DRG transduction

This experiment was designed to test the hypothesis that by suppressing the host's immune response by means of co-expressing mIL10 would allow transgene expression to proceed for at least as long as it did in the case of SCID mice. In order to test this $\approx 2x10^6$ pfu of either HSV1.pR20.9/UL43 or HSV1.pR20.9mIL10/UL43 vector were delivered via footpad inoculation to the DRGs of BALB/c mice (n=5, per vector per time point). The effect of mIL10 co-expression in the expression patterns of LacZ was examined at 2 weeks and 2 months post injection by counting the LacZ positive DRG neurons. The numbers achieved with either HSV1.pR20.9/UL43 (control) or HSV1.pR20.9mIL10/UL43 vectors are summarised in the table below.

BALB/c ONLY	Number of LacZ - expressing DRGs (Mean ±SEM)	
	2 weeks	2 weeks
HSV1.pR20.9/UL43	222 ± 97 (n=5)	11 ± 4 (n=5)
HSV1.pR20.9/UL43/mIL10	241 ± 83 (n=5)	154 ± 42 (n=5)

Table 7.3.3-a: HSV1.pR20.9mIL10/UL43 mediated LacZ expression in BALB/c DRGs.

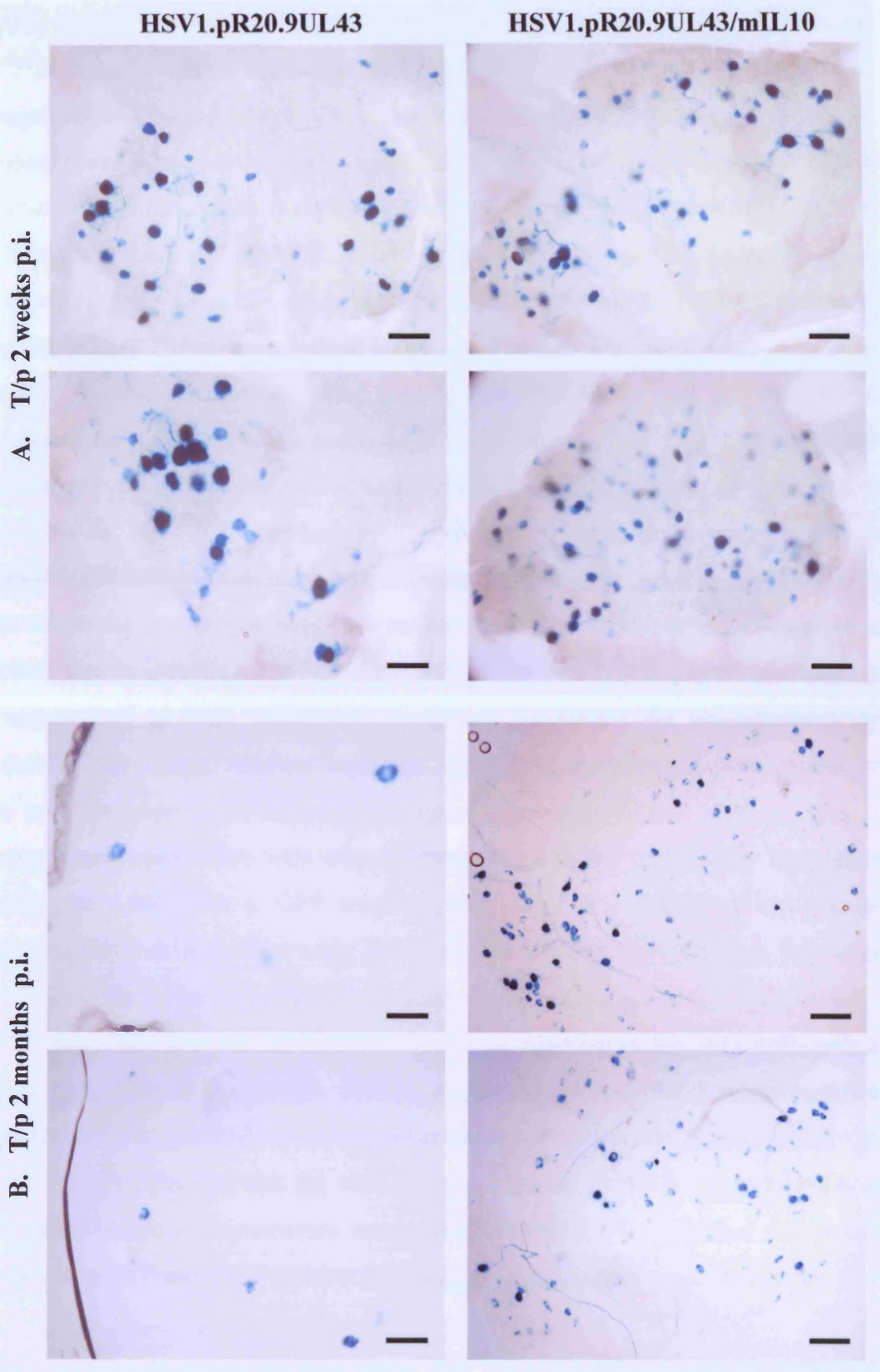
As shown in Figure 7.3.3-a, the presence of mIL10 is closely linked with the ability of the vector to support the long-term expression of the LacZ transgene. This effect becomes more pronounced in the 2-month time point when normally expression of the transgene would be confined to a handful of BALB/c DRGs. Table 7.3.3-a confirms this observation. Actual counts of the LacZ expressing DRGs demonstrate that in the case when LacZ is co-expressed with mIL10, the number of LacZ positive neurons at 2 months post injection is higher than the number noted in HSV1.pR20.9/UL43 transduced DRGs. Table 7.3.3-a demonstrates that at the 2-month time point expression of LacZ persists in 154±42 (n=5) DRG neurons in HSV1.pR20.9mIL10/UL43 treated animals. On the contrary only 11±4 (n=5) LacZ positive DRG neurons could be seen in HSV1.pR20.9/UL43 injected animals.

Figure 7.3.3-a: HSV1.pR20.9mIL10/UL43 LacZ expression at 2wks & 2months in BALB/c.

DRG neurons expressing LacZ in wholemounts of BALB/c mice DRG, 2 weeks (**A**) and 2 months (**B**) after vector injection into footpad. These figures together with Table 7.3.3-a show that the addition of mIL10 to the vector clearly leads to increased numbers of transduced DRGs at 2 months post-injection.

Scale bars: Left column (HSV1.pR20.9UL43 vector):
A: Top=150μm and Bottom=100μm
B: Top & Bottom=100μm;

Right column (HSV1.pR20.9 UL43mIL10 vector):
A: Top & Bottom=150μm,
B: Top & Bottom=200μm.


HSV1.pR20.9UL43
HSV1.pR20.9UL43/mIL10
A. T/p 2 weeks p.i.
B. T/p 2 months p.i.

7.4 Discussion

Even though the HSV1.pR20.9/UL43 vector used in this study is disabled for ICP34.5 and VP16 and can not cross trans-synaptically, it is still capable of some degree of replication. When the vector is delivered subcutaneously via foot-pad inoculation, it replicates locally and then it infects and establishes a latent and persistent infection within sensory ganglia (Palmer *et al.*, 2000). It is therefore plausible that HSV1.pR20.9/UL43 is capable of producing an immune response, especially prior to establishing latency. This is confirmed when inflammatory infiltrate was found surrounding motor neurons of the spinal cord following sciatic nerve inoculation with HSV1.pR20.9/UL43 (Perez *et al.*, 2004). Unfortunately, this study did not include any immune specific markers and hence the nature of the infiltrate cannot be established with certainty. It is not therefore possible to draw any conclusions regarding how this vector actually affects specific subclasses of immune cells such as satellite cells and how these differ to the responses invoked by wild type strains. This study however does support the notion that by disabling ICP34.5 and VP16, the stealth machinery of the vector is compromised as well. This would in theory accentuate the pro-inflammatory nature of the vector. Another argument supporting the potential involvement of an immune response in reducing transgene expression comes from a series of experiments where Vero cells were infected with a vector without any immediate early genes but with a GFP marker gene under the control of the human cytomegalovirus immediate early promoter (HCMVIEp). Even though this virus was not toxic to the host cells, expression of GFP faded gradually. Interestingly, GFP expression could be switched on by the re-infection of the same cells with a virus only deleted for ICP27. This is an indication that the genome remains functional in the infected cells long after transgene expression stops (Samaniego *et al.*, 1998). The fact that the viral genome remains active has been confirmed via super-infection experiments using an ICP4 and ICP27 disabled vector co-expressing GFP and LacZ *in vitro* (Lilley *et al.*, 2001b) (discussed in Chapter 1).

The experiments described here show that co-expression of mIL10 and LacZ from HSV1.pR20.9mIL10/UL43 appears to increase longevity of LacZ expression in transduced DRGs in comparison to the length of transduction

achieved with vectors co-expressing LacZ & GFP (i.e. control vector HSV1.pR20.9/UL43). This further supports the importance of the host immune response in clearance of the virally infected cells. It appears plausible that even though HSV1.pR20.9/UL43 is a minimally disabled vector, its ability to efficiently evade the host immune response is impaired enough to lead to its clearance and premature cessation of transgene expression. Additional supporting evidence for this hypothesis came after the completion of this study by Mogensen and colleagues who demonstrated that impaired VP16 activity via an *in1814* mutation leads to a hyper activation of pro-inflammatory cytokine production during HSV1 infection both *in vitro* and *in vivo* (Mogensen *et al.*, 2004). Hence, the induction of a Th2 response by the vector-encoded mIL10 appears to be able to compensate for the compromise in the stealth machinery of the virus caused by the *in1814* and 1764 mutations by dampening the host immune response. Even though the experiments carried out in this study targeted the PNS system it would be interesting to assess whether the CNS immune response also plays a part in the cessation of transgene expression noted with vectors of more severe disablement. IL10 would present a suitable means of re-enforcing the ability of highly disabled, CNS targeting vectors to persist for longer in infected neurons.

SUMMARY

The experiments described in this study support the hypothesis that the host's immune system is involved in the reduced, HSV1.pR20.9/UL43-mediated transgene expression noted in BALB/c DRG neurons. Suppressing the host's immune response using the HSV1.pR20.9mIL10/UL43 vector allows for transgene expression to be sustained for 2 months following footpad inoculation. This is a significant increase when one considers that the DRG transgene expression noted with vector HSV1.pR20.9/UL43 ceases at 2 weeks post inoculation in BALB/c mice. Overall the data presented here lead us to believe that Th1 immune responses contribute to the diminishing nature of transgene expression achieved with this vector. Co-expressing an immunomodulatory gene such as IL10 it is possible to significantly enhance the longevity of transgene expression achieved with HSV viral vectors via the peripheral route of administration.

CHAPTER 8.0

DISCUSSION

8.1 Summary

The studies included in this thesis set out to examine the use of replication incompetent HSV1 vectors as tools for the delivery of potentially therapeutic genes to the injured spinal cord and optic nerve. The experiments described in the previous chapters of this thesis have been discussed individually. In this section, the experimental findings are briefly summarised and final conclusions are drawn.

Replication incompetent HSV1 based vectors present a suitable gene therapy tool for use in the injured CNS (Palmer *et al.*, 2000; Lilley *et al.*, 2001b). The backbones employed here are minimally cytotoxic thus allowing the endogenous production of foreign genes by injured CNS neurons themselves. In Chapter 3, the capabilities and limitations of two of these vectors to deliver and sustain marker gene expression in the injured spinal cord were established. Vector HSV1.pR19CMV was selected as the backbone for the production of neurotrophin expressing constructs. This vector, despite its disablement, was shown to exploit the innate neurotropism of HSV1. A single spinal cord inoculation leads to the transduction of somata of neurons that project to the injection site. Importantly, the experiments of Chapter 3 showed that transgene delivery is equally efficient in the acutely and chronically injured spinal cord. Other virally based vector systems such as lentivirus, AV and AAV vectors, have been shown to be capable of some, albeit limited, retrograde axonal transport (Ghadge *et al.*, 1995; Kuo *et al.*, 1995; Mazarakis *et al.*, 2001; Kaspar *et al.*, 2002; Boulis *et al.*, 2003; Wong *et al.*, 2004).

The main limitation that came to light regarding the highly disabled HSV1.pR19CMV backbone was that it did not transduce corticospinal pyramidal neurons. This was probably due to the fact that these neurons are naturally refractory to HSV1 and are actively repressing expression from this vector. As discussed in Chapter 3, this feature is likely to be a reflection of the highly disabled nature of the vector and the fact that ICP0 levels are too low to overcome this repression. Further vector development in our laboratory has led to

the production of a new generation of less disabled vectors that can successfully transduce cortical pyramidal neurons following either direct cortical inoculation or a single spinal cord inoculation. These less disabled vectors do not bear deletions in the ICP34.5 and ICP27 genes (described in Chapter 3). In addition, in the closing stages of this project, these less disabled vectors were combined with an expression cassette that utilises the WPRE element responsible for stabilising transgene mRNAs and therefore potentially prolonging their expression (Chapter 6) (Paterna *et al.*, 2000; Klein *et al.*, 2002; Glover *et al.*, 2003; Hlavaty *et al.*, 2005).

The first growth factor expressing construct produced was the HSV1.pR19CMVrNT3 vector (Chapter 4). This vector was shown to be able to transduce non-complementing cells and thus enable them to secrete biologically active rNT3 protein. This was confirmed by the fact that the virally delivered rNT3 could promote the extension of a halo of neurites from E14 DRG explants. In the chronic injury model of CST lesion however, it was shown that the sole vector-mediated delivery of NT3 could enhance proximal sprouting but was not sufficient to promote regeneration of corticospinal axons. This finding is in line with previous reports showing that in order for any anatomically significant regeneration to occur via the delivery of a single neurotrophic factor, it may be necessary to deliver a biologically excessive amount of neurotrophin (Bradbury *et al.*, 1999). When the vector was used in combination with an E14 spinal cord segment transplanted within the lesion site, CST axons displayed enhanced sprouting proximally to the lesion and they were able to extend their axons ≈600 μm within the lesion site. This response was not observed when E14 transplants were not accompanied by NT3 vector administration. This finding is again in agreement with reports that have used this combinatorial approach (Bregman *et al.*, 1997; Bregman *et al.*, 1998). In the closing stages of this study a new vector became available. This vector employed the WPRE element and EF1α, a new promoter driving transgene expression instead of CMV. Inoculating the spinal cord this vector it is possible to selectively transduce large numbers of corticospinal neurons in layer V of the adult rat motor cortex. Furthermore, the corticospinal neurons remain strongly transduced up to 1 month post-inoculation.

This novel vector is therefore very promising for selective gene therapy of corticospinal neurons, which may be necessary to overcome their poor intrinsic regenerative capacity following spinal injury (manuscript submitted)

In Chapter 5, two new vectors were produced: HSV1.pR19CMVrBDNF and HSV1.pR19CMVmCNTF in order to examine their potential to induce a regenerative response in the injured rubrospinal tract. The combination of HSV1.pR19CMVrBDNF and HSV1.pR19CMVmCNTF did lead to a moderate increase in rubrospinal axon sprouting proximally to the site of a chronic rubrospinal tract lesion. Unfortunately, there was a lack of regeneration in the chronically injured rubrospinal tract even through sprouting was accompanied by the upregulation of SCG10 in sprouting axons. In addition, HSV1.pR19CMVrBDNF transduction of chronically injured red nucleus neurons led to some modest reduction in the extent of red nucleus cell body atrophy. However, this result can only be considered, in the context of a preliminary finding that will need to be repeated using a larger experimental group.

In Chapter 6, a panel of different neurotrophin-expressing vectors were examined in different combinations for their ability to promote regeneration in the crushed optic nerve injury model. These experiments examined the effect of transducing RGCs with a cocktail of neurotrophic factors containing rBDNF, mCNTF, rbFGF, rGDNF, rNT3 and NTN prior to inflicting an optic nerve crush. Different combinations of these vectors were used (Table 6.3.2-a). All combinations displayed some degree of enhanced sprouting proximally to the lesion site compared to control animals. In addition, injured RGCs can be seen extending their axons into the lesion as early as 20 days following the injury. In some cases, axons could be seen crossing the lesion site and entering the distal stump. This response was accompanied by enhanced GAP43 immunoreactivity in regenerating axons. Admittedly, transducing RGCs prior to inflicting the lesion does not represent the best simulation of the ability of these vectors to induce regeneration in a clinically relevant setting. This would be better achieved if delivery of the vector occurred after the lesion was carried out. This would of

course involve injecting the proximal stump of the optic nerve as the microtubules necessary for the neuronal trafficking mechanism are disrupted thus rendering viral retrograde transport impossible. This route of administration did not yield any RGC transduction with the fully disabled vectors. However, this route of delivery was found to be possible with the use of the new generation of less disabled, WPRE containing vectors (Figure 6.3.3-a & b). Widespread RGC transgene expression was seen following a single optic nerve injection. It will be interesting to explore the ability of these vectors to influence the regenerative response of axotomised RGCs. Neurotrophin expressing vectors based on this latest backbone have already been produced and characterised and are soon to be tested in such injury paradigms (not presented in this thesis).

The brief study outlined in Chapter 7 intended to explore whether counteracting the host's immune response could prolong transgene expression in virally transduced mouse DRG neurons. The vectors employed in this study were minimally disabled (HSV1.pR20.9UL43). Comparing marker gene expression in BALB/c and SCID mice demonstrated that these vectors were somewhat immunogenic, since transgene expression was prolonged in SCID animals. When these vectors were modified to co-express IL10 (HSV1.pR20.9UL43/mIL10), transgene expression in BALB/c mice was significantly enhanced. At the two-month time point, the number of HSV1.pR20.9UL43/mIL10 transduced BALB/c DRGs were comparable to the numbers transduced when the HSV1.pR20.9UL43 vector was used in SCID mice. The fact that it is possible to prolong transgene expression in DRG neurons with this approach is of importance in terms of future regeneration experiments. The fully disabled HSV1.pR19CMV backbone used in this thesis for direct CNS delivery is not able to sustain transgene expression in DRG neurons following peripheral inoculation (Perez *et al.*, 2004). However, with the development of this IL10 expressing vector it would be possible to combine direct delivery into the CNS (via the HSV1.pR19CMV vector) with the simultaneous transduction of DRG neurons.

8.2 Conclusion

Following trauma to the spinal cord a cascade of degenerative events is initiated. Although the events that follow are mechanically very destructive to CNS neurons there is considerable evidence to suggest that spinal cord neurons are capable of some limited axonal sprouting. However, this is rapidly aborted due to intrinsic and extrinsic inhibitory factors (reviewed in Chapter 1). For any regenerative approach to be successful it must therefore address both these parameters.

Despite their initial promise, the use of neurotrophic factors in regenerative studies has proved to be somewhat disappointing. Over the last decade, a number of reports have emerged that suggest that the sole administration of neurotrophins stimulated only minimal regeneration and in only a handful of axotomised spinal cord fibres (Ramer *et al.*, 2002; Zhou *et al.*, 2003; Lu *et al.*, 2004). In this thesis, the main approach used to induce regeneration in the injured corticospinal and rubrospinal tracts was based on the delivery of a single neurotrophic factor. Therefore, it is not surprising that the resulting regeneration was at best minimal. In the case of the crushed optic nerve however, where a cocktail of BDNF/CNTF/NTN or BDNF/CNTF/bFGF/GDNF was used, regeneration was notably enhanced. This combinatorial approach is in line with the findings of a recent study that demonstrated that a triple combination of neurotrophic factors (NT3, BDNF and bFGF) acts synergistically to enhance neuronal survival and axonal regeneration in axotomised RGCs (Logan *et al.*, 2006).

Over the last decade a wealth of accumulating evidence supports what is now considered a given by researchers: a multifaceted approach to regeneration is needed in order to maximise the limited potential of CNS neurons to regenerate (Thuret *et al.*, 2006). Based on this reasoning, it is very likely that the delivery of multiple neurotrophic factors (Lu *et al.*, 2004; Logan *et al.*, 2006) needs to be coupled with inactivation of a variety of growth inhibitory signals (Fawcett and Asher, 1999; Hunt *et al.*, 2002a; Sandvig *et al.*, 2004; Silver and Miller, 2004; Ahmed *et al.*, 2005a; Ahmed *et al.*, 2005b) in order for any functionally meaningful regeneration to be achieved. Indeed, experiments that have employed

this approach have shown significant promise in CST regenerative studies (von Meyenburg *et al.*, 1998; Logan *et al.*, 2002; Lu *et al.*, 2004; Fouad *et al.*, 2005; Lu *et al.*, 2007). The highly disabled HSV1.pR19CMV vectors used here, as well as the new generation of less disabled WPRE containing vectors, could potentially be used for delivering a combination of neurotrophins. In addition, these can be examined in a variety of combinations in order to identify the most regeneration-favourable mixtures. Their effects can be evaluated not only *in vivo* but also *in vitro*. This would allow for the identification and characterisation of specific molecular events governing their effects.

An alternative to transducing neurons to express neurotrophic factors would be to target molecules that inhibit regeneration. One such target would be the inhibition of small GTPases such as Rho. Many inhibitory factors signal through these pathways that ultimately affect the neuronal cytoskeleton. The vectors employed here could be used to knock down the expression of RhoA via constructing cassettes that produce a dominant negative version of this GTPase. Such a vector has already been produced and is currently under investigation. Another approach would be to use RNA silencing technology (Dykxhoorn *et al.*, 2006). RNA interference (RNAi) is an innate cellular process involved in post-transcriptional gene silencing. It involves a highly specific degradation of target mRNAs through the use of homologous, double stranded, 19-23*nt* RNA, termed small interfering RNA (siRNA). siRNAs essentially guide the intracellular RNA degradation machinery to target mRNAs in a highly specific manner (Shinagawa and Ishii, 2003). It can be easily generated from a single stranded RNA precursor that folds into a hairpin loop structure that is subsequently cleaved by the ribonuclease Dicer to produce siRNAs (Meister and Tuschl, 2004). The use of siRNA is currently under investigation by a variety of research laboratories and some reports as to its use in the injured CNS are beginning to emerge (Raoul *et al.*, 2006; Dykxhoorn *et al.*, 2006).

Overall, the vectors described here are powerful and versatile gene therapy tools that can be applied in either *in vivo* or *in vitro* paradigms designed to elucidate the mechanisms involved in the refractory nature of the injured CNS milieu.

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